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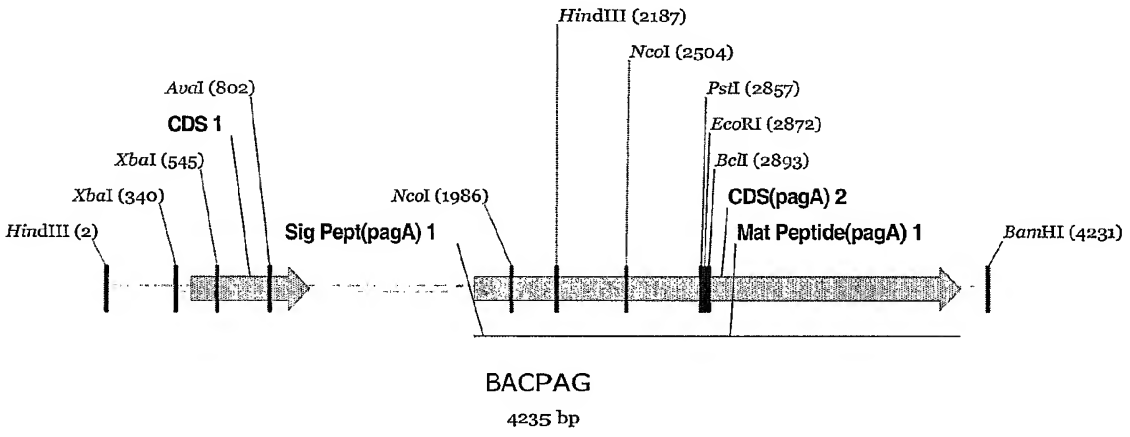

(57) Abstract: This invention provides DNA sequences that encode Bacillus anthracis Protective Antigen (PA) proteins that can be used for prophylactic immunization against anthrax infections. The codon usage in the sequence is optimized for expression in the yeast, including strains of Saccharomyces cerevisiae, a known safe fermentation source for vaccine products. The invention also includes vaccines and methods of vaccination against B. anthracis.

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TITLE OF THE INVENTION
ANTHRAX VACCINE

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefits of U.S. Provisional Applications Serial Number 60/470563 filed May 14, 2003.

FIELD OF THE INVENTION

The invention relates to the field of vaccination.

BACKGROUND

Anthrax infection, a disease caused by the spore-forming bacterium Bacillus anthracis, is highly lethal in the pulmonary or inhalation form (For general reviews see Friedlander, 2000; Little and Ivins, 1999; Mock and Fouet, 2001). With an increased awareness of the potential of this pathogen as a weapon, the need for a readily available, safe and effective vaccine for wide public use has grown (*See, e.g.*, Ibrahim *et al.*, 1999). Current vaccine preparations for human use are typically attenuated live spores or cell-free secretion products of B. anthracis adsorbed to alum (AVA). The former preparation was reportedly used in the Soviet Union and had a number of toxicities and production issues. In the US, AVA is reported to be used exclusively. Neither vaccine has the characteristics preferred for broad distribution in the event of an emergency or for general public prophylaxis, *i.e.*, long term protection, high level efficacy with limited number of immunizations, low reactogencity, and reliable, safe production. Thus, there is a need for new vaccines that provide a better fit to these criteria.

The cytotoxicity of germinating anthrax spores is mediated by two toxins, lethal factor and edema factor. Lethal factor (LF) is a 90-kD protein with catalytic activity which combines with an activated form of protective antigen, PA, an 83-kD protein, to produce an active toxin in macrophages. Edema factor is of similar molecular weight (89 kD) and combines in a separate complex with activated PA to enter its target cell, the neutrophil. Each of the three components are non-toxic individually.

Recent studies have shown that during infection, PA83 is cleaved by mammalian cells at amino acid position 167 of the mature peptide to yield a 63-kD component that binds to either lethal factor (LF) or edema factor (EF). Mutated PA83 that can not be cleaved is not functional for the production of toxin because it fails to form the heptameric pore that facilitates LF or EF to enter mammalian cells (Leppla, 2000). Under fermentation conditions and presumably during infection, B. anthracis synthesizes PA83 with a leader sequence that is responsible for its secretion into the medium and into tissues.

Although there are limited data in humans correlating protection against B. anthracis infection to specific immune responses, the available evidence suggests that neutralizing antibodies against the 83-

kD uncleaved protective antigen (PA83) is a surrogate marker for protective immunity (Baillie, 2001; Turnbull *et al.*, 1988). The data are more extensive in animal protection models using guinea pigs, rabbits, mice and rhesus monkeys (Fellows *et al.*, 2001; Ivins *et al.*, 1998). Immunization with purified PA or recombinant PA reportedly confers protection against challenge in rhesus macaques (Ivins *et al.*, 1998). Passive protection was also reportedly demonstrated with anti-PA antibodies. It is believed that there is a rough correlation between anti-PA titer and protection against lethal challenge with B. anthracis spores. Reuveny *et al.* reported a more quantitative correlate with neutralization of *in vitro* macrophage killing for the guinea pig model (Reuveny *et al.*, 2001). The cumulative evidence supports the concept that PA, whether obtained from B. anthracis or as a recombinant protein, can be an effective immunogen for anthrax prophylaxis. Published data also purport to show that the reactive epitopes are found within the cleaved, activated 63-kD form of PA (Flick-Smith *et al.*, 2002; Little and Ivins, 1999; Singh *et al.*, 1991).

One of the current anthrax vaccines is produced from a culture filtrate of germinating B. anthracis spores (Puziss, 1962; Puziss, 1963). The major component of this formulation is PA83 with some LF and EF. No further enrichment or purification of the protective component is reportedly performed. Minor, but highly potent reactogenic substances could also be present. Trace amounts of LF and EF purified from the B. anthracis fermentation could theoretically combine with PA cleaved after administration to yield toxins.

A variety of alternative preparations designed to address the toxicity issues raised above have been reported in the literature. These range from the use of attenuated B. anthracis strains with enhanced PA production, to acellular recombinant protein products to naked DNA preparations. A summary of these approaches are listed below:

Replicating vector strategies:

Bacillus anthracis – An attenuated, non-capsular strain has been reported that produces recombinant PA constitutively from a replicating vector. The heterologous constitutive promoter from α–amylase expressed as much as 10-fold higher titers of rPA than the native pagA promoter. The spores from the recombinant strains were stated to have elicited good anti-PA antibody titers and protected guinea pigs in a lethal challenge model (Cohen *et al.*, 2000). Appropriate containment for production of a live, attenuated spore vaccine is an important consideration for large-scale production.

Bacillus subtilis – This related non-pathogenic, sporulating bacillus is a good vehicle for expressing secreted proteins. It has been under investigation as a substitute production vehicle for production of PA for decades (Ivins and Welkos, 1986). The limitations of this system are safety concerns that require the development of non-sporulating strains and more importantly, degradation of PA by B. subtilis proteases (Baillie *et al.*, 1998a; Baillie *et al.*, 1998b). The maximal reported yield using this system was 2 mg/L. The introduction of mutations affecting catabolite repression and growth

phase regulation reportedly resulted in an increase in the yield of PA but a multiply protease-negative background to improve PA yield is still needed (Baillie *et al.*, 1998a).

Salmonella typhimurium – The PA gene was cloned into S. typhimurium with the aim of using the ability of this non-pathogenic (for humans) bacterium as a vector to introduce the PA antigen. A high copy plasmid with the p*lac*d promoter was used. The resultant strain was stated to have the ability to colonize the host and it was necessary to passage to regain colonization ability. The plasmid in the colonizing strain had lower copy number, suggesting that less PA antigen may be produced. This strain was reported to afford some protection against lethal challenge in mice (Coulson *et al.*, 1994).

Vaccinia virus – PA was cloned into vaccinia virus to use a replicating vector as an immunization strategy. Some protection was reported when using particular vector constructs (60% and 50% of mice and guinea pigs respectively), but anti-PA titers were produced only in mice (Iacono-Connors *et al.*, 1990). The relevance for humans is not known.

Lactobacillus casei – Lactobacilli are commensals of the gut and are generally regarded as safe (Zegers *et al.*, 1999). L. casei strains expressing PA internally have been reported with the aim of developing a live oral vaccine with a high level of mucosal immunity, an unproven strategy.

Acellular antigen production:

Baculovirus – The PA gene was cloned into baculovirus for the production of PA (Iacono-Connors *et al.*, 1990). The material produced was larger (86 kD vs. 83.5 kD) than authentic PA83 because it still had a secretory leader sequence. The preparations were reported to have produced high titer anti-PA responses in mice and to have protected mice and guinea pigs from lethal challenge when administered with an adjuvant. Production by baculovirus requires tissue culture growth – a costly protocol for large-scale production.

Escherichia coli – Several laboratories have reported production of recombinant wild-type PA in E. coli and have made mutated PA in this organism. One potential limitation of bacterially produced material is the requirement to purify the antigen from the lipopolysaccharide component of E. coli, an extremely potent pyrogen. Using the E. coli signal sequence on PA causes the material to accumulate in the periplasmic space (yield - 500 μg/L) (Sharma *et al.*, 1996). A his-tagged fusion accumulated as inclusion bodies and was denatured in urea and refolded (Gupta *et al.*, 1999). This change improved the yield to 2 mg/L. Until very recently, yields from inducible vectors were relatively low but constitutive overexpression has been reported to give yields of biologically active material at 125 mg/L (Chauhan *et al.*, 2001). Metal chelate chromatography was used for purification.

DNA plasmid – A DNA plasmid encoding PA63 was reported to have elicited a humoral immune response in mice. Mice immunized with the PA DNA vaccine were said to have been protected against lethal challenge with a combination of anthrax PA plus LF (Gu *et al.*, 1999).

Saccharomyces cerevisiae – Baker's yeast is generally recognized as safe for the production of heterologous protein. This yeast has been shown to be easily and rapidly cultivated under minimal

containment conditions and to lack toxic secreted or intracellular materials. Antigens isolated from yeast fermentations have good safety profiles. Small peptides and large proteins have been produced successfully. It has been proven to be a safe and commercially viable production vehicle for recombinant DNA derived hepatitis B vaccine. However, the current state of understanding of protein production in yeast does not allow one to predict which proteins will be made in sufficient quantities for commercial production. Variables that are not yet foreseeable or controllable are the degree of glycosylation, either O or N, the extent of protein degradation, and correct polypeptide folding. Potential glycosylation sites can be determined by sequence analysis, but the presence of such a consensus site does not guarantee that it will be glycosylated. Furthermore, addition of a yeast leader sequence does not by itself guarantee that a given protein will be secreted. Neither can one predict whether a protein will be toxic for the producing yeast cells. The parameters that are understood and can be controlled include the presence or absence of yeast non-translated leader sequences, mutations that reduce, but do not eliminate N-glycosylation, mutations that reduce protein degradation, promoter sequences that permit tightly regulated control of synthesis, plasmid copy number, and strain to strain variations. For any given protein, the outcome depends on the correct combination of sequence, expression vector, host strain, fermentation conditions and the intrinsic nature of the protein to be expressed, none of which can be predicted or guaranteed in the absence of empirical results. Thus, it is not possible to predict whether the sequence of a gene of interest will be translated into the desired functional protein in yeast or, if it is produced, whether it will be made in quantities sufficient for commercial application.

One report of a B. anthracis gene expressed in S. cerevisiae is a study designed to identify the substrates of the LF component via the yeast two-hybrid system (Vitale *et al.*, 1999). The experiment employed an inactive mutated LF as bait. This approach reportedly led to the identification of the MAP kinase kinases (MAPKKs) Mek1 and Mek2 as proteins capable of specific interaction with LF.

SUMMARY OF THE INVENTION

This invention relates to polynucleotides encoding a Bacillus anthracis Protective Antigen (also referred to herein as "PA") protein wherein the coding sequence of the polynucleotide has been codon-optimized for efficient expression in a yeast host cell. Preferably, the polynucleotides are DNA. In one preferred embodiment, the polynucleotide encodes a protein which retains its wild-type amino acid sequence. In one preferred embodiment the polynucleotide encodes a 63-kD form of the PA protein, referred to as PA63.

In another preferred embodiment, the polynucleotide encodes an 83-kD form of PA, referred to as PA83. In an alternate embodiment, the polynucleotides encode a mutated form of a PA protein which has reduced protein function as compared to wild-type protein, but which retains the immunogenicity of the wild-type protein such as the chymotrypsin processing site. A preferred mutated

form of the PA protein is a PA83 which has been mutated to prevent processing of the protein to the PA63 form of PA. This invention also relates to the PA proteins produced using the polynucleotides.

Another aspect of this invention is a vector carrying the polynucleotides encoding a codon-optimized PA protein. Yet another aspect of this invention are host cells containing these vectors.

In a preferred embodiment, the vector is a plasmid vector. In a particularly preferred embodiment, the vector is a plasmid vector comprising an insert wherein the insert comprises an expression cassette comprising:

a) a polynucleotide encoding a codon-optimized PA protein for expression in a yeast host cell; and

b) a promoter operably linked to the polynucleotide.

Another type of vector which is envisioned by this invention is a shuttle plasmid vector comprising a yeast origin of replication, a bacterial origin of replication and a polynucleotide insert, wherein the insert comprises an expression cassette comprising:

a) a polynucleotide encoding a codon-optimized PA protein for expression in a yeast host cell; and

b) a promoter which functions in yeast operably linked to the polynucleotide; and

c) a yeast-active transcriptional terminator.

An aspect of this invention is a yeast host cell carrying a polynucleotide of this invention. In a preferred embodiment, the polynucleotide is carried on a plasmid vector. In alternative embodiments, the polynucleotide is integrated into the chromosomal DNA of the yeast host cell. In a preferred embodiment, the synthetic PA gene is under the control of a promoter from the yeast galactose gene cluster (a GAL promoter) and the yeast host cell has been genetically engineered for controlled induction of genes regulated by GAL promoters. In alternative embodiments, the controlled induction is high level, mid level or low level induction of genes regulated by GAL promoters.

This invention also relates to a method of making a PA protein comprising expressing in a yeast host cell a polynucleotide encoding a PA protein, or mutated form of a PA protein which has reduced protein function as compared to wild-type protein, but which maintains immunogenicity, the sequence of the polynucleotide encoding PA comprising codons optimized for expression in a yeast host. In a preferred embodiment, the synthetic PA gene is under the control of a GAL promoter and the yeast host cell has been genetically engineered for controlled induction of genes regulated by GAL promoters. In a preferred method, the PA protein is produced as an intracellular product. In another preferred method, the PA protein is produced as a product secreted from the yeast host cell.

An aspect of this invention provides a vaccine against disease, cellular toxicity or death caused by B. anthracis. A vaccine of this invention includes an effective amount of a PA protein that was

expressed in yeast through the use of a polynucleotide of this invention. A vaccine of this invention also includes pharmaceutically acceptable excipients.

An aspect of this invention is a method of vaccinating a patient against disease, toxicity or death caused by B. anthracis. A vaccine of this invention is administered to a patient in a manner appropriate for the induction in the patient of an immune response against the PA protein of B. anthracis.

An aspect of this invention provides a method for purification of the PA protein expressed in yeast. In the method a homogenate of yeast cells is formed. Yeast proteases are inactivated in the homogenate by addition of a series of protease inhibiting compounds, and the PA protein is separated from yeast cellular proteins by a series of chromatographic separations.

An aspect of this invention is a process of isolating the 63 kilodalton form of Protective Antigen from a culture of yeast cells expressing a recombinant polynucleotide. In particular embodiments, the polynucleotide is carried on an expression vector in the cells, for example, a plasmid or artificial chromosome. The expression of the polynucleotide can be regulated by an inducible promoter. After expression of the polynucleotide, the yeast cells are disrupted to create a suspension which is subsequently clarified. The cells can be disrupted using methods known in the art including a press, homogenizer or microfluidizer. Clarification is commonly performed by low speed centrifugation. The clarified solution is mixed with a denaturant. In preferred embodiments the denaturant is a non-ionic denaturant, e.g., 6M urea. Thereafter the PA63 is purified from the solution by chromatography on cation exchange resin followed by chromatography on an anion exchange resin, in a denaturing buffer. In preferred embodiments, the PA63 is eluted from each column using a linear gradient of NaCl and 6M urea. The fractions containing the PA63 are then dialyzed against an appropriate buffer and, optionally, concentrated.

A polynucleotide according to this invention comprises a sequence encoding a B. anthracis PA protein, such coding sequence having been "codon optimized" for expression in yeast by substituting wild-type codons with codons that are preferred by a yeast host. Because the coding sequences used in this invention are not found in nature, we sometimes refer to polynucleotides having such codon optimized sequences as "synthetic" polynucleotides, as having a "synthetic" sequence encoding a PA protein, or simply as having a "synthetic" PA gene. In each case, the use herein of the term "synthetic" means that the gene has been modified so that it contains codons which are preferred for expression in a non-native host, e.g., a yeast or other eukaryote. In many cases, the amino acids encoded by the synthetic PA gene remain the same as the wild-type. In some embodiments, the synthetic gene may encode a modified protein.

The term "promoter" as used herein refers to a recognition site on a DNA strand to which the RNA polymerase binds. The promoter forms an initiation complex with RNA polymerase to initiate

and drive transcriptional activity. The complex can be modified by activating sequences termed "enhancers" or inhibiting sequences termed "silencers."

The term "cassette" refers to the sequence of the present invention which contains the nucleic acid sequence which is to be expressed and may also include regulatory sequences. Each cassette will have its own sequence. Thus by interchanging the cassette the vector will express a different sequence. Because of the restriction sites at the 5' and 3' ends, the cassette can be easily inserted, removed or replaced with another cassette.

The term "vector" refers to some means by which DNA fragments can be introduced into and maintained in a host organism or host tissue. Various types of vectors are known in the art including plasmid, virus (including adenovirus), bacteriophages, artificial chromosomes, linear DNA and cosmids. Vectors can replicate autonomously or integrate into the host cell genome.

The term "effective amount" means sufficient vaccine composition is administered to a patient so that an immune response results. One skilled in the art recognizes that this level may vary.

In reference to a nucleic acid or protein sequence, the term "native" means that the sequence is the same as it is found occurring in nature. It is also referred to as a "wild type" sequence.

The term "patient" means a mammal, particularly domesticated livestock including but not limited to dogs, cats, cows, bulls, steers, pigs, horses, sheep, goats, mules, donkeys, etc. Most preferably, a patient is a human.

BRIEF DESCRIPTIONS OF THE DRAWINGS

FIGS. 1A&B. FIG. 1A. Diagram of B. anthracis PA gene. FIG. 1B. DNA sequence (SEQ ID NO:47) and amino acid translation product (SEQ ID NO:48) of portion of B. anthracis PA83 gene corresponding to PA63.

FIGS. 2A&B. Yeast codon-optimized DNA sequence (SEQ ID NO:49) and amino acid translation product (SEQ ID NO:50) of synthetic B. anthracis PA63 gene.

FIGS. 3A&B. FIG. 3A. Yeast secretion vector pHK4α2 used for expression of secreted PA63. FIG. 3B. pAGT1 – pKH4α2 with PA63.

FIG. 4. Yeast expression vector pGAL110 used for internal expression of PA63 (PA63pGAL10#2).

FIG. 5. Time course of intracellular PA63 expression from transformant L-1 in S. cerevisiae strain 1849. Equal volumes of cell lysate were loaded on the gel. A and B indicate duplicate fermentations of transformant L-1 and vector control transformant K-1. Samples of L-1A and L-1B are shown for 0, 48, and 72 h in lanes 3-5 and 6-8, respectively. A 72 h sample of K-1A alone is shown in lane 2. A 48 h sample of K-1A and a 72 h sample of K-1B mixed with 10 ng PA63 standard are shown in lanes 1 and 9, respectively. Molecular size standards are also shown.

FIG. 6. An exemplary Western blot used for quantitation of internally expressed PA63 by transformant 8-3 (S. cerevisiae host strain 1260). Samples are from two independent experiments: lanes 12-15 are samples of cell lysates of 8-3 from one experiment, and lanes 4-7 and 8-11 represent duplicate sample preparations of cell lysates of 8-3 from another experiment. Replicate fermentations of 8-3 within the same experiment are represented by 4,5; 6,7; 8,9; 10,11; 12,13; 14,15. Odd and even-numbered lanes contain 0.25 and 0.5 μg total protein of 8-3 loaded, respectively. For the samples of the vector control transformant, 0.5 μg total protein was loaded. Lane 1 is a sample of vector control transformant 7-1A alone. Lanes 2, 3 and 16, 17 contain samples from vector control transformant 7-1A and -B mixed with 10 and 20 ng PA83, respectively. Molecular size standards are also shown.

FIG. 7. Time course for induction of secreted PA63 by strain 14A (S. cerevisiae host strain 1849). Equal volumes of supernatants were loaded in each lane. T0, T24, T48, and T72 are shown for 14A in lanes 1, 3, 5, and 7, respectively. T48 and T72 are shown for vector control transformant 12B alone in lanes 4 and 6. 10 ng PA63 standard is shown alone in lane 9 and mixed with vector control 12B from 24 h in lane 2. Lane 8 contains a supernatant from an independent 72 h fermentation of strain 14A. Molecular size standards are shown.

FIGS. 8A&8B. FIG. 8A. DNA sequence (SEQ ID NO:53) and FIG. 8B. Amino acid translation product (SEQ ID NO: 54) of native bacterial PA83 gene without its leader sequence.

FIG. 9. DNA sequence (SEQ ID NO: 51) and amino acid translation product (SEQ ID NO: 52) of yeast codon-optimized domain 4 of B. anthracis PA gene.

FIGS. 10A&B. FIG. 10A. DNA sequence (SEQ ID NO: 55) and FIG. 10B. Amino acid translation product (SEQ ID NO: 56) of yeast codon-optimized B. anthracis PA83 gene.

FIGS. 11A&B. FIG. 11A. FIG. 11A DNA sequence (SEQ ID NO: 57) and FIG. 11B. Amino acid translation product (SEQ ID NO: 58) of yeast codon-optimized B. anthracis PA83 with a mutation at the furin cleavage site.

FIG. 12. Exemplary Western blot comparing production of codon-optimized and native PA63 from transformants fermented for 72 h in complex YEHDG medium. Lane 1, MW size standards mixed with 100 ng cell-lysate protein from yeast transformant 8-3 which produces PA63 intracellularly (see Example 2). Lane 2, 20 ng purified E. coli-produced rPA83. Lanes 3-7 and 13 contain 25 μL medium supernatant from yeast transformants: lane 3, vector control transformant 12B; lanes 4 and 5 "native" PA63 transformants b-2 and b-3, respectively; lanes 6 and 7, "rebuilt" PA63 transformants 14A and 14C, respectively; lane 13, transformant 14A fermented in a previous experiment. Lanes 8-12 contain 500 ng protein cell lysate from yeast transformants: lane 8, 12B (vector control); lane 9, b-2; lane 10, b-3; lane 11, 14A; lane 12, 14C. Positions of molecular weight markers are indicated.

FIG. 13. Western blot depicting intracellular expression of uncleavable PA83 (lacking Furin cleavage site) from S. cerevisiae transformant Q-3 (yeast host 1558) and uncleavable PA83 from transformants 9-1 through 9-8 of yeast host strain 1260. Lanes 1-8 contain 4 μg cell lysate protein from transformants 9-1

through 9-8 and lane 9 contains 4 μg cell lysate protein from transformant Q-3. Lane 10 contains 1 μg of recombinant PA83 purified from E. coli.

FIG. 14. Western blot depicting intracellular expression of uncleavable PA83, from S. cerevisiae transformant 9-1, and cleavable PA83 from S. cerevisiae strain 5-C: lane 1, 200 ng E. coli-purified recombinant PA83; lanes 2-8 contain 250 ng of cell lysate protein; lane 2, vector control transformant fermented in shake flask; lanes 3 and 4, strain 9-1 (uncleavable PA83) fermented in shake flask; lanes 5 and 6, strain 5C (cleavable PA83) fermented in shake flask; lanes 7 and 8, strain 5C (cleavable PA83) fermented in culture tube.

FIG. 15. Western blot depicting intracellular expression of PA63 from large-scale fermentation of strain 8-3. Lanes 1 and 11: 10 ng PA63 mixed with 500 ng protein cell lysate of vector transformant 7-1. Lanes 2 and 12: 20 ng PA63 mixed with 500 ng protein cell lysate of vector transformant 7-1. Lanes 3 and 9: 10 ng PA83 mixed with 500 ng protein cell lysate from vector transformant 7-1. Lanes 4 and 10: 20 ng PA83 mixed with 500 ng protein cell lysate from vector transformant 7-1. Lanes 5 and 6, 250 and 500 ng protein of cell lysate, respectively from large-scale fermentation of 8-3. Lanes 7 and 8, 250 and 500 ng protein of cell lysate, respectively from tube fermentation of 8-3.

FIG. 16. Flow chart of a process for purification of PA63 protein from a yeast cell homogenate.

FIG. 17. SDS-PAGE gel analysis and Western blot of purified PA63 product and process retains. (A). SDS-PAGE analysis of 50HS chromatography; lane 1, load to 50HS column; lane 2, non-retained pool; lane 3, gradient fractions 12-21 (load to source Q); lane 4, gradient fractions 22-30; lane 5, high salt wash. (B). SDS-PAGE analysis of source Q chromatography. Lane 1, gradient fractions 17-18 (product); lane 2, gradient fractions 31-32; lane 3, gradient fractions 52-53.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a synthetic polynucleotide sequence that encodes for a PA protein of Bacillus anthracis. The invention also provides a synthetic polynucleotide sequence that encodes for an 83-kD form of the PA protein that is identical to the mature 83-kD PA protein of Bacillus anthracis or a 63-kD form of the PA protein. The invention also relates to synthetic sequences that encode mutated or truncated versions of PA63 or PA83 which, upon introduction into a patient, elicit antibodies that are protective against disease, cellular toxicity, debilitation or death due to anthrax infections or ameliorate such symptoms. These mutations include alterations that prevent the formation of activated PA63, such as alterations in the furin cleavage site.

The codon usage in the sequences for PA proteins have been optimized for expression in the yeast Saccharomyces cerevisiae, a known safe fermentation source for vaccine products. The invention also provides plasmids that are suitable for expression in yeast of the desired product intracellularly and

plasmids that are suitable for production in yeast of extracellular protein using a S. cerevisiae secretory leader sequence, an example of which is the alpha factor-based pre-pro-leader sequence. The invention also provides the 63-kD protein, PA63, the 83-kD protein PA83, and mutant proteins produced by expressing the synthetic DNA sequence in S. cerevisiae. The invention also includes methods of inducible and constitutive production of yeast produced PA.

The present invention provides a method for generating a polynucleotide sequence of B. anthracis PA that has been optimized for expression in yeast cells. In particular preferred embodiments, use of the polynucleotides results in the expression of authentic 63-kD PA as a secreted protein or as protein that accumulates within the yeast cell. It is readily apparent to those skilled in the art that portions of polynucleotide sequence can be used to generate larger PA proteins including polypeptides such as PA83 or smaller PA proteins that display domains of PA that induce an immunoprotective response in a patient. The polynucleotide sequence can also be used to create plasmids that will express PA in bacteria such as E. coli as native protein, or peptides derived from PA63. The bacterially expressed proteins can also be tagged proteins with groups such as 6X his, HA or GST, to facilitate purification. The present invention also provides a method for producing PA in recombinant yeast host strains which are genetically deficient in their ability to glycosylate proteins and/or carry a mutation in the *prb1* and or *pep4* protease genes.

The wild-type sequence containing the coding region of PA63 (BACPAG) is known. FIGs. 1 A and B. PA63 is a post-translational protein cleavage product of full-length PA83. In accordance with this invention, PA63 segments were converted to sequences having identical translated amino acid sequences, but with alternative codon usage as defined by Lathe (Lathe, 1985) and selected for yeast codons for highly expressed genes (Sharp and Cowe, 1991). The methodology may be summarized as follows:

1. Identify placement of codons for proper open reading frame.
2. Compare wild-type codon for observed frequency of use by yeast genes.
3. If codon is not one of those commonly employed by yeast, replace it with an optimal codon for high expression in yeast cells.
4. Repeat this procedure until the entire gene segment has been replaced.
5. Inspect new gene sequence for undesired sequences generated by these codon replacements (*e.g.* unwanted restriction enzyme sites, splice sites, promoters, undesirable palindrome or repeat sequences, etc.) and substitute codons that eliminate these sequences. See FIG. 2.
6. Assemble synthetic gene segments and test for expression in yeast cells.

Once the desired sequence is designed, the gene fragment encoding PA63 can be prepared by annealing and extension of oligomers designed to encode the final desired sequence.

Using the present invention, one can produce an immunogenic PA protein in a yeast host cell. It should be understood that the PA protein need not retain the functional activity of a wild-type

PA63 protein as produced by B. anthracis. The key concept is that the yeast produced protein is immunogenic and, when used as a vaccine, can stimulate an immune response that is fully or partially protective against the disease, debilitation, cellular toxicity or death caused by infection with B. anthracis. Therefore, because when making a PA protein of this invention, one is only concerned with retaining the immunogenic properties of the protein, and not the functional properties, one is free to modify the protein. Modified proteins can lack some or all of the functional characteristics of the wild-type PA63 protein and be equivalent, immunologically, to the wild-type protein. It can be useful to modify the protein in the process of designing yeast codon optimized versions of the protein, *i.e.*, changed amino acids, full length uncleavable PA83, or to produce PA63 protein that is less than full length.

It is known that epitopes within Domain 4 can stimulate antibodies against the PA63 protein that can protect against the disease, cellular toxicity, debilitation or death caused by anthrax infection. Therefore, when designing mutant forms of the PA protein, it is most preferred that one attempt to maintain the wild-type sequence of domain 4. Changes can be introduced in other areas of the PA protein, especially in the N-terminal domain between the N-terminus and the cleavage site for processing PA83 to the PA63 form of PA. In general, it can be useful to modify the PA protein sequence to prevent cleavage of the protein by proteases and/or protein processing enzymes in yeast and/or mammals. For example, one may choose to alter the amino acid sequence of the cleavage site located at the furin cleavage site, amino acids 163-167. This change is useful to prevent potential cleavage of the PA protein by the yeast Kex2 protease. Another example of a change is to alter the amino acid sequence of the chymotrypsin cleavage site located at amino acids 312-313.

Vectors

In accordance with this invention, a synthetic polynucleotide sequence encoding a PA protein can be inserted into a vector. The vector is preferably a plasmid, although other vectors including linear DNA linked to a promoter, integrative vectors, Yeast Artificial Chromosomes, phages or cosmid vectors may also be used. For convenience in manipulating the vector, the vector may be in a shuttle plasmid form, that is, may contain origins of replication for both yeast and bacterial hosts.

An expression cassette can be used. An expression cassette includes a synthetic sequence encoding a PA protein operably linked to a promoter and, optionally, other regulatory sequences such an enhancer or a terminator. The regulatory sequences are from yeast genes, are from non-yeast genes or are hybrid regulatory sequences derived by assembling regulatory elements from different organisms. In the latter two cases, the regulatory sequences are recognized by the protein and genetic expression systems of yeast.

It is preferred that the vector contain one or more promoters that are active in yeast or other host cells. For example, the vector could contain only a promoter active in yeast or, if expression is desired in another host, also or alternatively contain a promoter active in such host. Alternatively, a promoter could be used that is active in both yeast and an alternate host. In a preferred embodiments where expression is desired in yeast, the plasmid would contain a promoter that is active in yeast. Yeast promoters such as the ADH1, GAL1, GAL10, GAL7, PGK or GAP(TDH) promoters are commonly used in the art. However, numerous other promoters including other yeast promoters, eukaryotic promoters that are active in yeast and even hybrid promoters made from portions of promoters from two different organisms are known, and will become known. So long as the promoter is active in yeast, such promoters can be used interchangeably for expressing a synthetic PA gene in yeast. It is preferred that the synthetic codon-optimized sequence encoding the PA protein is operably linked to such a promoter.

In preferred embodiments, there are restriction sites flanking the expression cassette portion of the plasmid so that the cassette can easily be removed or replaced. The shuttle plasmid may be replicated in prokaryotic cells or eukaryotic cells.

Standard techniques of molecular biology for preparing and purifying DNA constructs enable the preparation of the plasmids, phages, shuttle plasmids and other recombinant nucleic acid molecules of this invention.

Expression of Synthetic PA Protein

A vector carrying a synthetic PA gene is introduced into a yeast host cell. Preferred yeast hosts include strains of Saccharomyces cerevisiae, Saccharomyces carlsbergensis and Kluyveromyces lactis. Other yeasts, including but not limited to Pichia pastoris, Hansenula sp., Schizzosaccharomyces pombe and Candidia sp. may also be useful. The cell is used to establish a yeast cell culture using standard techniques known in the art. To produce PA protein, the culture is incubated and allowed to ferment in an appropriate manner as known in the art. The synthetic PA gene can be expressed throughout the period of incubation or, preferably, the expression of the gene is induced at a particular time during the incubation. In the latter case, the time of induction can be chosen taking into consideration the age, cell density, metabolic state, growth phase or other measures or combinations of measures of the cell culture as deemed appropriate by the skilled artisan or as known and practiced in the art.

Production of PA using a synthetic DNA sequence can be strain-dependent and is expected to vary with culture conditions. Other known sources of variation in production are known to arise from mechanical conditions, local water and air. Therefore, one of skill in the art will be aware that when production of PA is performed using strains or culture conditions other than those exemplified

herein, one should perform routine testing and optimization of production conditions for the equipment and conditions employed.

The present methods can produce PA as an intracellular product or as a secreted product. Of the exemplified constructs, the constructs designed to produce secreted PA do not produce yields as large as the constructs designed to produce internal PA. However, it is believed that a secreted product will serve better in a production scale process because of ease of harvest and purification.

Recombinant Protective Antigen has been purified from various sources (E. coli, B. subtilis) in the 83-kilodalton form using both native and denaturing purification processes. We are not aware of literature reports on the purification of the 63-kilodalton form of protective antigen.

Once the synthetic PA gene has been expressed for an appropriate period of time the PA protein can be harvested from the culture using standard methods of protein isolation and purification known and practiced in the art. If the PA was expressed in a fashion that produced secreted PA protein, the protein is isolated from the culture fluid. If all or a portion of the protein exists in refractile or inclusion bodies within yeast cells, the protein can be isolated, denatured and refolded using standard techniques.

If recombinant protein is produced and retained in an intracellular compartment of the host yeast cell, a cell pellet can be isolated from the culture medium by centrifugation, washed, and lysed by a variety of known physical or chemical methods. The crude lysate can be further clarified to remove cellular debris components. Purification of recombinant protein is undertaken from either the supernatant liquid or the pellet, depending on the solubility of the expressed protein. A variety of known techniques including, but not limited to, membrane filtration, centrifugation, chromatography, and differential extraction may be employed to purify the recombinant protein from host cell contaminants. In the case of poorly soluble or aggregated proteins, a variety of known techniques may be employed to improve solubilization, including, but not limited to, detergent extraction, solvent extraction, and use of denaturants and refolding techniques.

Formulations

The vaccine of the present invention can be formulated according to methods known and used in the art. Guidelines for pharmaceutical administration in general are provided in, for example, *Modern Vaccinology*, Ed. Kurstak, Plenum Med. Co. 1994; *Remington's Pharmaceutical Sciences* 18th Edition, Ed. Gennaro, Mack Publishing, 1990; and *Modern Pharmaceutics* 2nd Edition, Eds. Banker and Rhodes, Marcel Dekker, Inc., 1990.

PA protein of the present invention can be prepared as acidic or basic salts. Pharmaceutically acceptable salts (in the form of water- or oil-soluble or dispersible products) include conventional non-toxic salts or the quaternary ammonium salts that are formed, *e.g.*, from inorganic or organic acids or

bases. Examples of such salts include acid addition salts such as acetate, adipate, alginate, aspartate, benzoate, benzenesulfonate, bisulfate, butyrate, citrate, camphorate, camphorsulfonate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, fumarate, glucoheptanoate, glycerophosphate, hemisulfate, heptanoate, hexanoate, hydrochloride, hydrobromide, hydroiodide, 2-hydroxyethanesulfonate, lactate, maleate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, oxalate, pamoate, pectinate, persulfate, 3-phenylpropionate, picrate, pivalate, propionate, succinate, tartrate, thiocyanate, tosylate, and undecanoate; and base salts such as ammonium salts, alkali metal salts such as sodium and potassium salts, alkaline earth metal salts such as calcium and magnesium salts, salts with organic bases such as dicyclohexylamine salts, N-methyl-D-glucamine, and salts with amino acids such as arginine and lysine.

It is preferred that the adjuvant is chosen as appropriate for use with the particular protein used as well as the ionic composition of the final formulation. Consideration should also be given to whether the PA protein alone will be formulated into a vaccine or whether the protein will be formulated into a combination vaccine. In the latter instance one should consider the buffers, adjuvants and other formulation components that will be present in the final combination vaccine.

Aluminum based adjuvants are commonly used in the art and include aluminum phosphate, aluminum hydroxide, aluminum hydroxy-phosphate and aluminum hydroxy-sulfate-phosphate. Trade names of adjuvants in common use include ADJUPHOS, MERCK ALUM and ALHYDROGEL. The conjugate can be bound to or co-precipitated with the adjuvant as desired and as appropriate for the particular adjuvant used.

Non-aluminum adjuvants can also be used if approved for use in the expected patient population. Non-aluminum adjuvants include QS21, Lipid-A and derivatives or variants thereof, Freund's complete or incomplete adjuvant, neutral liposomes, liposomes containing vaccine, microparticles and cytokines or chemokines.

It is preferred that the vaccine be formulated with an aluminum adjuvant. In other preferred embodiments, the vaccine is formulated with both an aluminum adjuvant and QS21.

It is preferable, in certain embodiments, to formulate the PA protein with immunogens from Haemophilus influenza, hepatitis viruses A, B, or C, human papilloma virus, measles, mumps, rubella, varicella, influenza virus, polio virus, smallpox, rotavirus, Streptococcus pneumoniae and Staphylococcus aureus. Combination vaccines have the advantages of increased patient comfort and lower costs of administration due to the fewer inoculations required.

When formulating combination vaccines one should be mindful of the various buffers and adjuvants used with the other immunogens. Some buffers may be appropriate for some immunogen-adjuvant pairs and not appropriate for others. In particular, one should assess the effects of phosphate levels on the various immunogen-adjuvant pairs to assure compatibility in the final formulation.

Vaccination

The vaccine of the present invention can be administered to a patient by different routes such as intravenous, intraperitoneal, subcutaneous, intranasal or intramuscular. A preferred route is intramuscular. Suitable dosing regimens are preferably determined taking into account factors well known in the art including age, weight, sex and medical condition of the subject; the route of administration; the desired effect; and the particular PA protein and formulation employed. The vaccine can be used in multi-dose vaccination formats. It is expected that a dose would consist of the range of 1 μg to 1.0 mg total protein. In an embodiment of the present invention the range is 0.1 mg to 1.0 mg. However, one may prefer to adjust dosage based on the amount of protein delivered. In either case these ranges are guidelines. More precise dosages should be determined by assessing the immunogenicity of the PA protein produced so that an immunologically effective dose is delivered. An immunologically effective dose is one that stimulates the immune system of the patient to establish a level immunological memory sufficient to provide long term protection against disease, cellular toxicity, debilitation or death caused by infection with B. anthracis. The PA protein is preferably formulated with an adjuvant.

The timing of doses depend upon factors well known in the art. After the initial administration one or more booster doses may subsequently be administered to maintain antibody titers. An example of a dosing regime would be day 1, 1 month, a third dose at either 4, 6 or 12 months, and additional booster doses at distant times as needed.

A patient or subject, as used herein, is a mammal, particularly domesticated livestock and animals including but not limited to dogs, cats, cows, bulls, steers, pigs, horses, sheep, goats, mules, donkeys, etc. Most preferably a patient is a human. A patient can be of any age at which the patient is able to respond to inoculation with the present vaccine by generating an immune response. The immune response so generated can be completely or partially protective against disease, cellular toxicity, debilitation or death caused by infection with B. anthracis.

The following examples are offered by way of illustration and are not intended to limit the invention in any manner.

EXAMPLE 1:

Design and Synthesis of a Synthetic PA Sequence

The wild-type sequence for the PA gene encoding PA63 as part of PA83 (BACPAG) is known (See FIGs. 1A and B). In accordance with this invention, PA63 segments were converted to sequences having identical translated amino acid sequences, but with alternative codon usage as using the methodology described above. The yeast codon-optimized sequence is shown in FIGs. 2A and B.

The gene fragment encoding PA63 was prepared by the annealing and extension of 24 oligomers (91-110 bp in length) designed to encode the final desired sequence.

5'GACGTCGACGGTGTAACCTTCGACTTCCAAGGAGTCTGGAATACCGTCGTTGTCTCTGTCTGGAACAG TTGGACCAGCGGAGGTGGA (SEQ ID NO:1)

5'CCTTGGAAGTCGAAGGTTACACCGTCGACGTCAAGAACAAGAGAACCTTCTTGTCTCCATGGATCTCC AACATTCACGAAAAGAAGGGTTTGACCAAG (SEQ ID NO:2)

5'CGGTGACCTTTTCGAAGTCGGAGTATGGGTCGGAAGCAGTAGACCACTTTTCTGGGGAAGACTTGTAC TTGGTCAAACCCTTCTTTTCGTGAATGTTGGAG (SEQ ID NO:3)

5'CCGACCCATACTCCGACTTCGAAAAGGTCACCGGTAGAATCGACAAGAACGTCTCCCCAGAAGCTAGA CACCCATTGGTTGCTGCTTACCCAATTGTCCACGT (SEQ ID NO:4)

5'CTGGTTTCGGAGTCCGTGTTTTGGGTGGATTGGTCTTCGTTCTTGGACAAAATGATGTTTTCCATGTC AACGTGGACAATTGGGTAAGCAGCAACCAATGG (SEQ ID NO:5)

5'ATCCACCCAAAACACGGACTCCGAAACCAGAACTATCTCTAAGAACACCTCCACTTCTAGAACCCACA CTTCCGAAGTCCACGGTAACGCTGAAGTTCACGC (SEQ ID NO:6)

5'CGATAGCGACGGTGGAGGAGTTGGAGTTGGAGAAACCAGCAGAGACGGAACCACCAATGTCGAAGAAA GAAGCGTGAACTTCAGCGTTACCGTGGACTTCG (SEQ ID NO:7)

5'CTCCAACTCCAACTCCTCCACCGTCGCTATCGACCACTCTTTGTCCTTGGCTGGTGAAAGAACTTGGG CTGAAACCATGGGTTTGAACACTGCTGACACCGC (SEQ ID NO:8)

5'GGAGGTGGTTGGCAAGACGTTGTAGATTGGAGCGGTACCAGTGTTGACGTATCTAATGTTAGCGTTCA ATCTAGCGGTGTCAGCAGTGTTCAAACCCATGG (SEQ ID NO:9)

5'CTCCAATCTACAACGTCTTGCCAACCACCTCCTTGGTCTTGGGTAAGAACCAAACCTTGGCTACTATC AAGGCTAAGGAAAACCAATTGTCCCAAATCTTGGC (SEQ ID NO:10)

5'GGAGAAGTCGTCTTGAGCGTTCAAAGCGATTGGAGCCAAGTTCTTGGATGGGTAGTAGTTGTTTGGAG CCAAGATTTGGGACAATTGGTTTTCCTTAGCC (SEQ ID NO:11)

5'CCAATCGCTTTGAACGCTCAAGACGACTTCTCCTCTACTCCAATCACCATGAACTACAACCAATTCTT GGAATTGGAAAAGACTAAGCAATTGCGTTTGGACACC (SEQ ID NO:12)

5'CCGGTGTCGACTCTGACTCTACCGTTTTCGAAGTTGTAAGTAGCAATGTTACCGTAAACTTGGTCGGT GTCCAAACGCAATTGCTTAGTCTTTTCCAATT (SEQ ID NO:13)

5'CTTCGAAAACGGTAGAGTCAGAGTCGACACCGGTTCCAACTGGTCTGAAGTCTTGCCACAAATCCAAG AAACCACCGCTAGAATCATCTTCAACGGTAAGGACTTG (SEQ ID NO:14)

5'GTCTGGCTTGGTGGTTTCCAATGGGTCGGATGGGTTGACAGCAGCAATTCTTCTTTCAACCAAGTTCA AGTCCTTACCGTTGAAGATGATTCTAGCGGTGG (SEQ ID NO:15)

5'CCGACCCATTGGAAACCACCAAGCCAGACATGACCTTGAAGGAAGCTTTGAAGATCGCTTTCGGTTTC AACGAACCAAACGGTAACTTGCAATACCAAGGTAAGG (SEQ ID NO:16)

5'GCCAATTGGTTCTTGATGTTTTGAGAGGTTTGTTGGTCGAAGTTGAAGTCGAATTCGGTGATGTCCTT ACCTTGGTATTGCAAGTTACCGTTTGGTTCG (SEQ ID NO:17)

5'CCAACAAACCTCTCAAAACATCAAGAACCAATTGGCTGAATTGAACGCTACCAACATTTACACTGTTT TGGACAAGATCAAGTTGAACGCCAAGATGAACATCTTGATCAGAGAC (SEQ ID NO:18)

5'GGGCTTCCTTGACAACGGATTCGTCAGCACCGACAGCAATGTTGTTTCTGTCGTAGTGGAATCTCTTG TCTCTGATCAAGATGTTCATCTTGGCGTTCAACTTG (SEQ ID NO:19)

5'GCTGACGAATCCGTTGTCAAGGAAGCCCACAGAGAAGTCATCAACTCCTCCACCGAAGGTTTGTTGTT GAACATCGACAAGGACATCAGAAAGATCTTGTCCGG (SEQ ID NO:20)

5'GAGGAAATGTTCAACATGTCGTATCTGTCGTTGATGACTTCCTTCAAACCTTCGGTGTCTTCAATTTC GACGATGTAACCGGACAAGATCTTTCTGATGTCCTTGTCGATG (SEQ ID NO:21)

5'GTCATCAACGACAGATACGACATGTTGAACATTTCCTCTTTGAGACAAGACGGTAAGACCTTCATCGA CTTCAAGAAGTACAACGACAAGTTGCC (SEQ ID NO:22)

5'GGGTTGATGATGGTGTTTTCCTTAGTGACAGCGTAAACGTTGACCTTGTAGTTTGGGTTGGAAATGTA CAATGGCAACTTGTCGTTGTACTTCTTGAAGTCGATGAAGG (SEQ ID NO:23)

5'CGCTGTCACTAAGGAAAACACCATCATCAACCCATCCGAAAACGGTGACACTTCCACCAACGGTATCA AGAAGATTTTGATCTTCTCTAAGAAGGGTTACGAAATTGGT (SEQ ID NO:24)

The oligomers were alternating, overlapping sense and antisense sequences that spanned the entire length of the optimized PA63 coding sequence. Each oligomer had a complementary overlap of 28-38 bp with the adjoining oligomer (duplex had a Tm of 72-78 C° as calculated by VECTOR NTI program, version 7 INFORMAX, Bethesda, MD).

Six separate extension reactions were performed using four adjoining overlapping oligomers and sense and antisense PCR primers (28-32 nt in length with duplex Tm=62-68 C°). Appropriate conditions for PCR are those described in International Publication number WO 01/14416A2. As a result of these PCR reactions, 6 colinear fragments of the gene were created (referred to herein for convenience as C+, D, E, F, G, H).

The fragments were gel-separated on agarose and the appropriately-sized products excised were purified using the GENE CLEAN Spin Column method (QBIOgene, Carlsbad, CA) as recommended by the manufacturer. Colinear fragments C+, D, and E and colinear fragments F, G, and H were combined in subsequent PCR reactions with the appropriate primers to yield fragments II+ and III, respectively. The complete gene for PA63 was then assembled by an additional PCR reaction in which fragments II+ and III were combined using distal sense and antisense primers which incorporated *Bam*HI sites to allow cloning of the final product into a yeast vector. The sense primer incorporated additional upstream sequences to permit in frame cloning with a yeast secretion leader including its cleavage site via the Kex2 protease. The resulting 1.7-kb product (designated PA63 +L) was gel-isolated and cloned into pCR-Blunt II-TOPO (INVITROGEN, Carlsbad, CA) as recommended by the manufacturer. The sequence was obtained and errors identified. The gene was subcloned into pGEM3zf+ (PROMEGA, Madison, WI) and the errors sequentially corrected by site-directed mutagenesis using the QUIK-CHANGE Site-directed Mutagenesis Kit (STRATAGENE, La Jolla, CA) using the manufacturer's recommendations. A final PCR reaction with HIGH FIDELITY PCR Supermix (INVITROGEN, Carlsbad, CA) using an antisense primer
(5'CGCGGATCCTTAACCAATTTCGTAACCCTTCTTAGAGAA) (SEQ ID NO: 25)
which incorporated a stop codon upstream of the *Bam*HI site and a sense primer which contained a *Bam*HI site as listed below (SEQ ID NO:26) plus a convenient portion of the gene sequence amplified the corrected sequence The reaction product was subcloned into a TA cloning vector (INVITROGEN, Carlsbad, CA) using the manufacturer's protocol.

The DNA was purified from the final clone and the *Bam*HI fragment subcloned in the proper orientation into a yeast secretion vector (pHK4α2, FIG. 3) to construct pAGT1. The sequence of the insert was verified by DNA sequencing.

It is readily apparent to those skilled in the art that this invention extends to the use of DNA sequence data and its deduced amino acid sequence from naturally occurring modifications of the PA protein and from recombinant sequences that confer improved properties such as increased

immunogenicity or genetically engineered weapons. For secretion, the initial vector is pKH4α2, which utilizes the yeast *GAL10* promoter and contains a unique *Bam*HI site for fusion (in-frame) of the ORF of interest to the yeast alpha factor (MFα1) pre-pro secretory leader. The PA63 5'-end was modified to add the last 7 amino acids of the pro-leader including the cloning site and Lys-Arg cleavage site for the yeast Kex2 protease by using PCR amplification with appropriate primers as described above. The 5'-region is shown below:

*Bam*HI KEX2 site

5'-*GTCACGG* ATC CTG TCT TTG GAT AAG AGA 3' (SEQ ID NO: 26)

Ile Leu Ser Leu Asp Lys Arg (SEQ ID NO: 27)

It is also readily apparent to those skilled in the art that for expression of the PA protein, any suitable yeast-active leader sequence that permits suitable expression levels in yeast and extracellular secretion may be substituted for the pre-pro leader sequence. Similarly, any suitable yeast-active transcriptional terminator may be substituted for *ADH1*. Plasmid pAGT1 was used to transform *leu2* yeast strains to Leu$^+$. Immunoblot analysis of cell lysates and cell supernatants prepared from the transformed cells verified the expression of a protein with approximately the expected size which reacted with a monoclonal antibody directed against PA83 (BIODESIGN INTERNATIONAL, Saco, ME.).

EXAMPLE 2:

Intracellular Expression of PA63 in Yeast

The DNA encoding the PA63 post-translational protein product, and with codons optimized for yeast expression was amplified from the final corrected clone as carried in the TA vector described in Example 1 using the following sense and antisense primers, respectively, 5' CGCGGATCC**CACAAAACAAA - ATG** TCCACCTCCGCTGGTCCAACTGTTCC3' (SEQ ID NO: 28) and 5'CGCGGATCCTTAACCAATTTCGTAACCCTTCTTAGAGAA3' (SEQ ID NO:29) containing a *Bam*HI restriction site and TAA as a stop codon. The *Bam*HI fragment was subcloned in the proper orientation into a yeast vector (pGAL110, FIG. 4) to construct PA63pGAL10#2. The sequence of the insert was verified by DNA sequencing. The plasmid was used to transform *S. cerevisiae* strains #1260 (*MATa, leu2-2, 112, mnn9, ura3Δ, can1, his3Δ::GAL10p-GAL4-URA3, prb1Δ::HIS3,* cir°), #1558 (*MATa, leu2-04, prb1Δ::HIS3, mnn9::ura3, ade1, his3::URA3,* cir°) and #1849 (*MATa, leu2-04, prb1Δ::HIS3, mnn9::ura3, ade1, his3::GAL10p-GAL4-URA3,* cir°) to leucine prototrophy (Leu$^+$) by using a spheroplast transformation protocol (Hinnen *et al.*, 1978). Transformants were selected on synthetic agar medium lacking leucine and containing 1 M sorbitol. The synthetic medium contained per

liter of distilled water: agar, 20 g; sorbitol, 182 g; and SD medium minus leucine, 27.4 g (QBIOgene #4811-015) (Carlsbad, CA). Clonal Leu^+ isolates were obtained by serial growth on Leu^- plates.

Five-ml seed cultures were grown at 30° C in 5X minus leucine medium containing 4.0 % glucose and 0.1 M sorbitol medium for 18-24 h to OD_{600} of 1.5-3.0/ml. 5X minus leucine medium contains the following components per liter: Yeast Nitrogen Base without added amino acids or ammonium sulfate, 8.5 g; adenine, 0.40 g; L-tyrosine, 0.25 g; uracil, 0.20 g, succinic acid, 10.0 g, ammonium sulfate, 5.0 g and 50 ml of Leucine-Minus Solution #3. Leucine-Minus Solution #3 contains per liter of distilled water, L-arginine, 2 g; L-histidine, 1.0 g; L-isoleucine, 6 g; L-lysine 4.0 g; L-methionine, 1.0 g; L-phenylalanine, 6.0 g; L-tryptophan, 4.0 g. A 0.3 ml aliquot was transferred to either 5.0 ml of 5X minus leucine medium containing 2 % glucose, 4 % galactose or YEHDG medium for 72 h to a final OD_{600} of 5-16.0/ml. YEHDG medium contains per liter: L-Hy-Soy peptone-Sheffield, 10 g; Yeast extract, 20 g; L-dextrose, 16 g; D(+) galactose, 40 g.

After harvesting a total of 10 ODs per sample, the cell pellets were broken with glass beads in 0.3 mL lysis buffer (0.1 M sodium phosphate buffer, pH 7.2, 0.5 M NaCl ,1 mM PMSF). The lysate was recovered by centrifugation, the unbroken cells/beads were washed with 0.3 mL of lysate buffer and the clarified supernatants were combined. The clarified lysate was assayed for protein by the BIO-RAD Protein Assay Dye Reagent system (cat#500-0006) (BIORAD, Hercules, CA) according to the manufacturer's instructions. The cell lysates were analyzed for the expression of PA63 by immunoblot analysis after electrophoresis on either 4-15 % gradient Tris-HCl gels (BIORAD #161-1158) or 10-20% gradient Tris-HCl Criterion gels (BIORAD # 345-0043) in 1X Tris glycine SDS buffer (cat#161-0732) (BIORAD, Hercules, CA) under reducing and denaturing conditions. When equal amounts of protein lysate were subjected to electrophoresis, the samples contained 0.25-0.5 μg of total cellular protein. Sometimes equal volumes of cell lysate were analyzed and in this case, 0.05-1.0 μg total protein were loaded on the gels. The gels were electroblotted onto 0.2 micron PVDF membrane filters. To estimate protein size, prestained standards between 10 and 250 kDa were run in parallel with the lysates (cat # 161-0372) (BIORAD, Hercules, CA).

Recombinant PA83 prepared from E. coli BL21 as described by Roberts, J.E. et al. (Roberts *et al.*, 1998) was used as size standard. PA63 was derived from PA83 by treatment with trypsin as described by Miller et al. (Miller *et al.*, 1999) using Trypsin, type XI (SIGMA # T1005) (SIGMA CHEMICAL CO., St. Louis, MO) and Trypsin soybean inhibitor (SIGMA #T9003) (SIGMA CHEMICAL CO., St. Louis, MO). Either PA63 or PA83 were run as standards on every gel. For both qualitative and quantitative purposes, PA63 and PA83 standard were mixed with cell lysates prepared from a vector control transformant. Proteins were immunodetected using a monoclonal antibody to PA83 obtained from BIODESIGN (Clone C3 #C86613M) (BIODESIGN INTERNATIONAL, Saco, ME) as primary antibody and goat anti-mouse IgG (H+L) horseradish peroxidase-linked whole antibody (ZYMAX #81-6520) (ZYMED LABORATORIES, South San Francisco, CA, City, State) as the

secondary antibody. The filters were processed using the "WESTERN LIGHTNING™ Chemiluminesence Reagent Plus kit (PERKIN ELMER, Wellesley, MA).

A time course of induction of PA63 by transformant L-1 (strain1 849) fermented in duplicate, A and B, in complex YEHDG medium showed a major protein band that had the same mobility as the PA63 standard (FIG. 5). No expression was detected with a vector control transformant, K-1A (lane 2). Optimal expression was obtained at 48 hours (lanes 4 and 7). Production of internal PA63 was greater in complex medium than in a defined medium. Isolate 8-3 was chosen as the best producer of internally-produced PA63 in yeast strain 1260 based on Western analysis using PA63 and PA83 as standards and ELISAs using PA83 as standard.

EXAMPLE 3:

Quantitation of Internal Yields

To estimate the yield of PA per culture volume, 5 mL culture of strain 8-3 which produces PA63 intracellularly, and vector control transformant 7-1 (both transformants of strain 1260) were processed either for quantitative ELISAs or for semi-quantitative Westerns. Two independent fermentation experiments designated one and two, were conducted with duplicate fermentations, A and B, for each transformant. For each experiment, the samples were analyzed by both techniques using authentic recombinant PA83 from E. coli as standard. ELISA and Westerns were performed using standard techniques. The antibodies used to capture and detect PA63 in the ELISA were monoclonal antibodies to PA83 (clone BAP0103, #C86301M,) and (clone C3, #C86613M,), respectively, (BIODESIGN INTERNATIONAL, Saco, ME). The antibody used in the Western blots was clone C3. A typical Western blot for quantitation is shown in FIG. 6. The results from ELISA determinations and semi-quantitative Westerns from the two independent experiments with culture 8-3 are shown in Table 1. These yields are within the range of the best yields reported for PA production from B. anthracis (Leppla, 1988) and recombinant PA production in E. coli (Chauhan *et al.*, 2001) without the potential of contamination with toxic bacterial components.

Table 1 μg PA/mL culture (tube)
-transformants of 1260

Analysis Method	Isolate 8-3[a]titer(μg PA/mL)
Experiment 1	
ELISA-1[b]	71
ELISA-2[b]	58
Western-1	47
Western-3	52
Experiment 2	
ELISA-3[b]	144
Western-2	99
Western-3	82
Average[c]	79

[a]Avg. of duplicate fermentations-Avg. variation, 1.4 and 1.2-fold for ELISA and Western, respectively

[b] Underestimate due to inhibition of PA detection by cell lysate

[c] Average of all values from both experiments

EXAMPLE 4:

Expression of Secreted PA63 in Yeast Strain 1849

The synthetic DNA encoding the PA63 protein and with codons optimized for yeast expression was purified from the clone described in Example 1 and the *Bam*HI fragment subcloned in the proper orientation into a yeast secretion vector (pHK4α2, FIG. 3) to construct pAGT1. The sequence of the insert was verified by DNA sequencing. For secretion, the vector of choice is pKH4α2, which utilizes the yeast *GAL10* promoter and contains a unique *Bam*HI site for fusion (in-frame) of the ORF of interest to the yeast alpha factor (MFα1) pre-pro secretory leader. The 5'-end of the synthetic PA63 was modified to add the last 7 amino acids of the pro-leader including the cloning site and Lys-Arg cleavage site for the

yeast Kex2 protease by using PCR amplification with appropriate primers. The 5'-region is shown below:

*Bam*HI KEX2 site

5'-*GTCAC*GG ATC CTG TCT TTG GAT AAG AGA (SEQ ID NO:26)

Ile Leu Ser Leu Asp Lys Arg (SEQ ID NO:30)

Plasmid pAGT1 was used to transform S. cerevisiae strains #1260 (MATa, leu2-2,112 mnn9, ura3Δ , can1, his3Δ::GAL10p-GAL4-URA3, prb1Δ::HIS3, cir°), #1558 (MATa, leu2-04, prb1Δ::HIS3, mnn9::ura3, ade1, his3::URA3, cir°) and #1849 (MATa, leu2-04, prb1Δ::HIS3, mnn9::ura3, ade1, his3::GAL10p-GAL4-URA3, cir°) to leucine prototrophy (Leu^+) by using a spheroplast transformation protocol (Hinnen et al., 1978). Transformants were selected on synthetic agar medium lacking leucine and containing 1 M sorbitol. The synthetic medium contained per liter of distilled water: agar, 20 g; sorbitol,182 g; and SD medium minus leucine, 27.4 g (QBIOgene #4811-015)(QBIO, Carlsbad, CA). Clonal Leu^+ isolates were obtained by serial growth on Leu^- plates.

Five-ml seed cultures were grown at 30° C in 5X minus leucine medium containing 4.0 % glucose and 0.1 M sorbitol medium for 18-24 h to OD_{600} of 1.5-3.0/mL. 5X minus leucine medium contains the following components per liter: Yeast Nitrogen Base w/o amino acids or ammonium sulfate, 8.5 g; adenine, 0.40 g; L-tyrosine, 0.25 g; uracil, 0.20 g, succinic acid, 10.0 g, ammonium sulfate, 5.0 g and 50 ml of Leucine-Minus Solution #3. Leucine-Minus Solution #3 contains per liter of distilled water, L-arginine, 2 g; L-histidine, 1.0 g; L-isoleucine, 6 g; L-lysine 4.0 g; L-methionine, 1.0 g; L-phenylalanine, 6.0 g; L-tryptophan, 4.0 g. A 0.3 mL aliquot was transferred to either 5.0 mL of 5X minus leucine medium containing 2 % glucose, 4 % galactose or YEHDG medium for 72 h to a final OD600 of 5-16.0/ml. YEHDG medium contains per liter: L-Hy-Soy peptone-Sheffield, 10 g; Yeast extract, 20 g; L-dextrose, 16 g; D(+) galactose, 40 g.

The yeast cells were removed and the supernatants were directly analyzed for the expression of PA63 by immunoblot analysis. Twenty-five microliter samples were subjected to electrophoresis on 4-15 % gradient Tris-HCl gels (cat #161-1158)(BIORAD, Hercules, CA) in 1X Tris glycine SDS buffer (cat #161-0732) (BIORAD, Hercules, CA) under reducing and denaturing conditions. The gels were electroblotted onto 0.2 micron PVDF membrane filters. To estimate protein size, prestained standards between 10 and 250 kDa were run in parallel with the samples (cat # 161-0372) (BIORAD, Hercules, CA). Recombinant PA83 from E. coli BL21 as described by Roberts, J.E. et al. (Roberts *et al.*, 1998) was used as size standard. PA63 was derived from PA83 by treatment with trypsin as described by Miller et al. (Miller *et al.*, 1999) using Trypsin, type XI (cat # T1005)(SIGMA CHEMICAL CO., St. Louis, MO) and Trypsin soybean inhibitor (cat #T9003) (SIGMA CHEMICAL CO., St Louis, MO). Either PA63 or PA83 were run as standards on every gel. For both qualitative and quantitative purposes, PA63 and PA83 standard were mixed with cell lysates prepared from a vector control transformant.

Proteins were immunodetected using a monoclonal antibody to PA83 obtained from BIODESIGN INTERNATIONAL, Saco, ME(Clone C3 #C86613M) as primary antibody and goat anti-mouse IgG (H+L) horseradish peroxidase-linked whole antibody (Zymed #81-6520) (ZYMED LABORATORIES, South San Francisco, CA) as the secondary antibody. The filters were processed using the "WESTERN LIGHTNING™ Chemiluminesence Reagent Plus kit (PERKIN ELMER, Wellesley, MA).

Multiple isolates were screened using small scale shake flask fermentations or roller tube fermentations. Culture 14A in strain 1849 was determined to yield the most secreted PA63 as determined by semi-quantitative Western blots. A time course of production of secreted PA from strain 14A is shown in FIG.7. A protein band of ~75 kDa was detected minutely at 24 h, increased at 48 h, and was maximally produced at 72 h (see lanes 3, 5 and 7, respectively). No protein was detected in the supernatant of a transformant containing the vector control alone, 12B, shown at 48 and 72 h as shown in lanes, 4 and 6, respectively. There were some smaller proteins that could be degradation products. These proteins appear as early as 24 h and appear to get smaller with time. The ~75-kDa protein is larger than the PA63 control. There are several possibilities for the altered mobility of the secreted protein including N- or O-linked core glycosylation and uncleaved leader sequence. A protein band of ~75 kDa was detected in all three of the transformed *S. cerevisiae* strains tested. Production of secreted PA was greater in complex medium than in a defined medium. Assays on cellular lysates indicated that some PA63 was retained intracellularly.

PA63 secretion was not improved in 2 different *pep4* mutants (deficient in vacuolar proteases) incubated for various times.

EXAMPLE 5:

Domain 4 Construct

Native PA63 has a cellular receptor binding site within domain 4 that has been implicated in vaccine-induced immunity. Domain 4, a 140-amino acid protein corresponding to bacterial amino acids 596-735 of SEQ ID NO: 54, is capable of inducing antibodies that protect mice from infection with B. anthracis (Flick-Smith *et al.*, 2002). Further evidence that domain 4 has a key role was shown by neutralization of toxin with affinity-enhanced recombinant antibodies directed to an epitope within domain 4. Thus, it can be readily seen that expression of recombinant domain 4 in yeast would yield a small protein in a safe expression host. It is expected that this domain and other appropriate immunogenic domains of PA expressed in yeast would offer the advantage of increased immunogenicity, easier purification and higher yields than the entire protein.

The wild-type sequence for the gene encoding the uncleaved PA83 (BACPAG) is known (See FIG. 8). In accordance with this invention, domain 4 sequence without the bacterial signal sequence having identical translated amino acid sequences, but with alternative codon usage as defined by Lathe (Lathe, 1985) and selected for yeast codons for highly expressed genes (Sharp and Cowe, 1991) was

amplified by PCR from a plasmid containing PA63 DNA sequence optimized for yeast codon usage. The 5' primer (GTCACGGATCCTGTCTTTGGATAAGAGATTCCACTACGACAGAAACAACATT) (SEQ ID NO: 31)
contained the desired yeast leader sequence for secretion and the 3' primer
(CGCGGATCCTTAACCAATTTCGTAACCCTTCTTAGAGAA) (SEQ ID NO: 32)
contained a *Bam*HI restriction site and TAA as a stop codon. The PCR fragment was digested with *Bam*HI and cloned into secretory vector. The yeast codon optimized domain 4 sequence without the leader is shown in Figure 9A and its translation in 9B.

EXAMPLE 6:

PA83 Construct and Expression

The wild-type sequence for the gene encoding the uncleaved PA83 (BACPAG) is known (See FIG.8). In accordance with this invention, PA83 segments without the bacterial signal sequence were converted to sequences having identical translated amino acid sequences, but with alternative codon usage as defined by Lathe (Lathe, 1985) and selected for yeast codons for highly expressed genes (Sharp and Cowe, 1991). See FIG. 11. The methodology was the same as for Example 1.

Native, mature PA83 binds to cellular receptors and requires activation by endoproteolytic cleavage to interact with either LF and EF to form active toxin (Reviewed by Mock and Fouet, 2001). A mutated form of PA83 that has the amino acid sequence RKKK (SEQ ID NO: 33) instead of the wild-type RKKR (SEQ ID NO: 34) sequence within the furin cleavage site at amino acids 164-167 does not form active toxins with EF and LF (Beauregard et al, 2000). Proteolytic cleavage is rate-limiting for internalization of the protein and the mutated protein remains on the surface. Yet this mutated protein still contains the immunoprotective domains implicated in vaccine-induced immunity such as domain 4 (Flick-Smith *et al.*, 2002). This mutant form of PA83 and other appropriate immunogenic domains of PA expressed in yeast can offer the advantages of increased immunogenicity, easier purification and higher yields.

The gene fragment encoding PA83 with a mutation of R to K at amino acid 167 to alter the furin and potential Kex2p cleavage site was prepared by the annealing and extension of 31 oligomers (91-110 bp in length) designed to encode the final desired sequence. The oligomers were alternating, overlapping sense and antisense sequences that spanned the entire length of the optimized PA83 coding sequence. Each oligomer had a complementary overlap of 28-38 bp with the adjoining oligomer (duplex had a Tm of 72-78 C° as calculated by Vector NTI program, version 7, INFORMAX, Bethesda, MD). Eight separate extension reactions were performed using four adjoining overlapping oligomers and sense and antisense PCR primers (28-32 nt in length with duplex Tm=62-68 C°). The actual conditions of PCR were similar to those described in International Publication number WO 01/14416A2.

The oligomers and resulting fragments D,E,F,G, and representing the PA63 portion of the molecule are the same as in Example 1. The N-terminal portion were created from the following oligomers which were used to create fragments A, B and C. The 12 oligomers for A, B, and C are the following:

5'GAAGTTAAGCAAGAAAACAGATTGTTGAACGAATCTGAATCCTCTTCCCAAGGTTTGTTGGGTTA
CTACTTCTCCGACTTGAACTTCCAAGCTCCAATGGTCGTCACC-3' (SEQ ID NO: 35)

5'CGGATGGGATGTTCTCTAATTCGGAGGATGGGATGGACAAGTC
ACCGGTAGTAGAGGAGGTGACGACCATTGGAGCTTGGAAGTTCAAGTCGG-3' (SEQ ID NO: 36)

5'TCCCATCCTCCGAATTAGAGAACATCCCATCCGAAAACCAATACTTCCAATCCGCTATCTGGTCC
GGCTTCATCAAGGTCAAGAAGTCCGACG-3' (SEQ ID NO: 37)

5'GGAAGCCTTGTTGATGACTTCTTGGTCGTCGACCCACATAGTAACGTGGTTGTCAGCGGAGGTAG
CGAAAGTGTATTCGTCGGACTTCTTGACCTTGATGAAGCCGG-3' (SEQ ID NO: 38)

5'GGGTCGACGACCAAGAAGTCATCAACAAGGCTTCCAACTCCAACAAGATCAGATTGGAAAAGGGT
AGATTGTACCAAATTAAGATCCAATACCAAAGAGAAAACCCAACCG-3' (SEQ ID NO: 39)

5'GAGATGACTTCCTTCTTGTTTTGGGAGTCGGTCCAGTACAACTTGAAGTCCAAACCCTTTTCGGT
TGGGTTTTCTCTTTGGTATTGGATCTTAATTTGG-3' (SEQ ID NO: 40)

5'GGACCGACTCCCAAAACAAGAAGGAAGTCATCTCYTCTGACAACTTGCAATTGCCAGAATTGAAG
CAAAAGTCYTCCAACTCCAGAAAGAAGAAGTCCACCTCCGCTGG (SEQ ID NO: 41)

5'GACGTCGACGGTGTAACCTTCGACTTCCAAGGAGTCTGGAATACCGTCGTTGTCTCTGTCTGGAA
CAGTTGGACCAGCGGAGGTGGActtCTTCTTTCTGGAGTTGG-3' (SEQ ID NO: 42)

5'CCTTGGAAGTCGAAGGTTACACCGTCGACGTCAAGAACAAGAGAACCTTCTTGTCTCCATGGATC
TCCAACATTCACGAAAAGAAGGGTTTGACCAAG-3' (SEQ ID NO: 43)

5'CGGTGACCTTTTCGAAGTCGGAGTATGGGTCGGAAGCAGTAGACCACTTTTCTGGGGAAGACTTG
TACTTGGTCAAACCCTTCTTTTCGTGAATGTTGGAG-3' (SEQ ID NO: 44)

5'CCGACCCATACTCCGACTTCGAAAAGGTCACCGGTAGAATCGACAAGAACGTCTCCCCAGAAGCT
AGACACCCATTGGTTGCTGCTTACCCAATTGTCCACGT-3' (SEQ ID NO: 45)

5'CTGGTTTCGGAGTCCGTGTTTTGGGTGGATTGGTCTTCGTTCTTGGACAAAATGATGTTTTCCAT
GTCAACGTGGACAATTGGGTAAGCAGCAACCAATGG-3' (SEQ ID NO: 46)

As a result of these PCR reactions, the following 8 fragments of the gene were created: A, B, C, D, E, F,G, H. Fragment C contains the mutation which results in a lysine in place of the arginine at amino acid 167.

The fragments were gel separated on agarose with the appropriately-sized products excised and purified using the Gene Clean Spin Column method (QBIOGENE, Carlsbad, CA) as recommended by the manufacturer. Fragments A and B, fragments C, D, and E, and fragments F, G, and H were combined in a subsequent PCR reaction with the appropriate primers to yield the fragments I, II and III, respectively. The complete gene for PA83 was then assembled by an additional PCR reaction in which

fragments I, II and III were combined using distal sense and antisense primers which incorporated *Bam*HI sites to allow cloning of the final product into a yeast vector. The sense primer incorporated additional upstream sequences that permit in-frame cloning with a yeast secretion leader and its Kex2 protease cleavage site as in Example 1. The resulting 2.2-kb product (designated PA83 U +L) was gel isolated and cloned into pCR-Blunt II-TOPO (INVITROGEN, Carlsbad, CA) as recommended by the manufacturer. The sequence was obtained and errors identified. The final DNA sequence and encoded protein sequence are shown in Figure 11. The gene was subcloned into pGEM3zf+ (PROMEGA, Madison, WI) and the errors sequentially corrected by site-directed mutagenesis using the Quik-Change Site-directed Mutagenesis Kit (STRATAGENE, La Jolla, CA) using the manufacturer's recommendations. It can be readily seen by those skilled in the art that the sequence of the PA83 can be changed to produce wild-type and altered forms of PA83 in response to mutated versions made by man or naturally occurring modifications.

EXAMPLE 7:

Preparation of Immunogenic Compositions

Purified PA63 is formulated according to known methods, such as by the admixture of pharmaceutically acceptable carriers, stabilizers, or a vaccine adjuvant. The immunogenic PA63 of the present invention may be prepared for vaccine use by combining with a physiologically acceptable composition such as, e.g. PBS, saline or distilled water. The immunogenic PA63 are administered in a dosage range of about 0.1 to 100 mcg, preferably about 1 to about 20 mcg, in order to obtain the desired immunogenic effect. The amount of PA63 per formulation may vary according to a variety of factors, including but not limited to the individual's condition, weight, age and sex. Administration of the PA63 formulation may be by a variety of routes, including but not limited to oral, subcutaneous, topical, mucosal and intramuscular.

An antimicrobial preservative, *e.g.* thimerosal, optionally may be present. The immunogenic antigens of the present invention may be employed, if desired, in combination with vaccine stabilizers and vaccine adjuvants. Typical stabilizers are specific compounds, e.g. polyanions such as heparin, inositol hexasulfate, sulfated beta- cyclodextrin, less specific excipients, e.g. amino acids, sorbitol, mannitol, xylitol, glycerol, sucrose, dextrose, trehalose, and variations in solution conditions, e.g. neutral pH, high ionic strength (ca. 0.5-2.0M salts), divalent cations (Ca^{2+}, Mg^{2+}). Examples of adjuvants are $Al(OH)_3$, $Al(OH)_x(SO_4)_y(PO_4)_z$ and $Al(PO_4)$. The vaccine of the present invention may be stored under refrigeration or in lyophilized form.

EXAMPLE 8:

S. cerevisiae codon usage required for optimal production of PA63 in yeast

To determine whether the alteration of codons used in Examples 1-4 provided for optimal production in yeast, the authentic bacterial sequence of PA63, which is the 1.7-kb furin cleavage product of authentic native PA83, was amplified by PCR from authentic native bacterial PA83 isolated from B. anthracis. The authentic native bacterial PA83 had been cloned and the sequence subsequently corrected so that the amino acid sequence matched that of codon-optimized PA83. The native PA63 sequence as inserted into the same yeast secretory expression vector as the codon-optimized sequence (see Example 4). The amount of secreted and internal PA63 was determined by semi-quantitative Western analysis.

DNA from B. anthracis ATCC strain 14578 was obtained from a 5.0-mL culture. The culture was inactivated and total DNA was purified by the addition of 0.5 mL 20 % SDS, 5.0 mL of phenol-chloroform and 0.75 mL 5.0 M NaCl. The DNA was purified by standard procedures and resuspended in TE for use in PCR.

The full-length PA83 (2.2 kb) was amplified with *Bam*HI restriction enzyme sites by PCR using the following primers (*Bam*HI sites underlined): 83 sense, 5'CGCGGATCCGAAGTTAAACAGGAGAACCG3' (SEQ ID NO: 59), 83 antisense - 5'CGCGGATCCTTATCCTATCTCATAGCCTT 3'(SEQ ID NO: 60).

Two rounds of PCR were used to amplify the desired regions. The first round used PFU turbo DNA polymerase, genomic DNA according to the manufacturer's recommendations and the following PCR conditions: 1 min 95 °C, 1 X; 95 °C 45 sec, 55 °C 45 sec, 72 °C 2.5 min 30X; 72 °C 10 min, 1X; 4 °C hold. The second round was performed with 1 µL of product from the first round, and PCR SUPERMIX HIGH FIDELITY (cat # 10790-020)(INVITROGEN, Carlsbad, CA) according to the manufacturer's recommendations. The PCR conditions were as follows: 30 sec 94° C, 1 X; 94° C 30 sec, 55° C, 30 sec, 68° C, 2.5 min, 25X; 68° C, 7 min, 1X; 4° C hold. The PCR product was purified and concentrated on a Gene Clean Spin column according to the manufacturer's recommendation (QBIOGENE, Carlsbad, CA). The PCR products were cut with *Bam*HI, purified by Gene Clean Spin columns and ligated to the vector pKH4alpha2 that had been digested with *Bam*HI and treated with alkaline phosphatase to prevent self-ligation. The orientation of the insert was confirmed by restriction digest and the DNA sequence of plasmid pba83PAG#11 analyzed. Four bp that differed from the published sequence, used to construct the yeast codon-optimized version, were found.

To facilitate correction of the sequence, the *Bam*HI fragment of pba83PAG#11 was gel isolated and concentrated on a Gene Clean Spin column according to the manufacturer's recommendation (QBIOGENE, Carlsbad, CA). The *Bam*HI fragment was ligated to pGEM3zf that had been digested with *Bam*HI and treated with alkaline phosphatase to construct pba83#13. The sequence was corrected using QUICK CHANGE MULTI-SITE-DIRECTED mutagenesis kit (STRATAGENE, La Jolla, CA) according to the manufacturer's instructions and the DNA sequence of the resultant plasmid, pba83multi#5, was verified.

In order to clone the 1.7-kb furin cleavage product of authentic native PA83, and to facilitate cloning into the yeast secretory vector with the appropriate leader sequence, the following sense and antisense primers (*Bam*HI sites underlined), respectively, 5'CGCGGATCCTGTCTTTGGATAAGAGAAGTACAAGTGCTGGACCTAC3' (SEQ ID NO: 61) and 5'CGCGGATCCTTATCCTATCTCATAGCCTT3' (SEQ ID NO: 62) were used for PCR with pba83multi#5 as template. The PCR conditions were as described above using PCR SUPERMIX HIGH FIDELITY (cat # 10790-020)(INVITROGEN®, Carlsbad, CA). The *Bam*HI fragment was isolated as described above and cloned into pCR2.1TOPO (INVITROGEN, Carlsbad, CA) according to the manufacturer's recommendation. Subsequently, the *Bam*HI fragment was gel-isolated again and ligated into pKH4α2 that had been digested with *Bam*HI and treated with alkaline phosphatase. The correct orientation and sequence was confirmed by DNA sequencing of plasmid pba63pKH4α2#11.

The plasmid pba63pKH4α2#11 was used to transform S. cerevisiae strain #1849 as in Examples 2 and 4. Leu $^{+}$ transformants obtained with pba63pKH4α2, pAGT1 (codon-optimized PA63, Example 4), and pKH4α2 (vector control) were fermented in complex medium YEHDG as described in Examples 2 and 4. Supernatants of cultures of twenty-four independent Leu^{+} transformants obtained with pba63pKH4α2 were screened for the production of secreted PA63 as described in Example 4. No PA63 was detected from any of the transformants containing the authentic native sequence, even after prolonged exposure of the blot. Secreted PA63 was however, detected from transformants containing the yeast codon-optimized sequence (data not shown).

We had previously found both secreted and intracellular PA63 produced by transformants containing the plasmid pAGT1, designed to secrete codon-optimized PA63 (Example 4). To ensure that PA63 was not produced inside the cell from a transformant containing "authentic native" PA63 sequence, we evaluated selected transformants for both intracellular and extracellular production of PA63. Fermentations and assessment of PA63 production were conducted as described in Examples 2 and 4. The amount of PA63 produced in yeast from the authentic native bacterial sequence was compared to that of the "codon-optimized" gene by Western blot analysis as shown in FIG. 12. PA63 was detected in both the medium supernatants and cell lysates of transformants containing the codon-optimized gene as shown in lanes 6, 7 and 11, 12, respectively. In both cases, the PA63 detected was larger than 63 kDa. It was also larger than PA63 produced from a transformant constructed to produce PA63 intracellularly (see Example 2) as shown in lane 1 and described in Example 4. In contrast, PA63 was not detected in either cell lysates (lanes 9 and 10) or medium supernatants (lanes 4 and 5) from transformants containing the "native" construct, even after a very long exposure of the blot. No protein was detected in either supernatants (lane 3) or cell lysates (lane 8) of the vector control transformant.

EXAMPLE 9:

Intracellular Expression of Uncleavable PA83 with Altered Furin Cleavage Site in S. cerevisiae

The DNA encoding the PA83 protein with codons optimized for yeast expression was amplified from the final corrected clone (lacking the Furin cleavage site) as carried in the pGEM3Zf+ vector described in Example 6 using the following sense and antisense primers, respectively, 5'CGCGGATCCCACAAAACAAAATGGAAGTTAAGCAAGAAAACAGA 3' (SEQ ID NO: 63) and 5'CGCGGATCCTTAACCAATTTCGTAACCCTTCTTAGAGAA3' (SEQ ID NO: 29) containing a *Bam*HI restriction site and TAA as a stop codon. The *Bam*HI fragment was subcloned in the proper orientation into a yeast vector (pGAL110, FIG. 4) to construct PA83pGAL10#24. The sequence of the insert was verified by DNA sequencing. The plasmid was used to transform S. cerevisiae strains #1260 (*MATa, leu2-2, 112, mnn9, ura3Δ, can1, his3Δ::GAL10p-GAL4-URA3, prb1Δ::HIS3,* cir°), #1558 (*MATa, leu2-04, prb1Δ::HIS3, mnn9::ura3, ade1, his3::URA3,* cir°) and #1849 (*MATa, leu2-04, prb1Δ::HIS3, mnn9::ura3, ade1, his3::GAL10p-GAL4-URA3,* cir°) to leucine prototrophy (Leu^+) as described in Examples 2 and 4.

For screening multiple transformants of strains 1849 and 1558, small-scale trial fermentations were conducted in complex YEHDG medium and evaluated for expression of PA83 by Western blot as described in Example 2. A major protein that co-migrated with the E. coli PA83 standard was detected by Western blotting in cell lysates of transformants of both strains 1849 and 1558. No proteins were detected in lysates of vector control transformants. The best internal producer of PA83 was a transformant of 1558, strain Q-3, with a volumetric productivity of 12.0 μg PA83 produced per mL of culture medium with PA83 comprising 7.0 % of the total cell protein. Production of internal PA83 was greater in complex than in a defined medium. Media compositions were described in Example 2.

Production of uncleavable PA83 was evaluated from strain Q-3 and several transformants of strain 1260, 9-1 through 9-8, that were fermented in 50 mL of medium in 250-mL shake flasks. Inoculum development began by thawing the frozen vials and transferring 1.0 mL inoculum to 50 mL of medium contained in a 250-mL flask. Strain Q-3 was transferred into 5X minus leucine + 4% Dextrose medium and strains 9-1 through 9-8 were transferred into 5X minus leucine minus adenine + 4% Dextrose medium. The composition of 5X minus leucine medium was described in Example 2. The flasks were incubated at 28° C and agitated at 250 rpm. For each strain, samples were removed aseptically after ~ 24 h to determine the glucose concentration and OD_{600}.

When the glucose in the 250-mL flask reached between 5 and 25 g/L, 1.0 mL of culture was transferred to another 250-mL Erlenmeyer flask containing YEHDG medium. YEHDG medium was described in Example 2. Again, the flasks were incubated at 28° C and agitated at 250 rpm. For each strain, samples were removed aseptically after 48 h and 72 h to determine the glucose and galactose concentration and OD_{600}.

The flasks were harvested after 72 h and pellets corresponding to 25 OD_{600} units were collected for each strain. The cell-pellets were broken with 0.5 g of 0.45 mm acid washed glass beads in 0.3 mL of 0.2 M HEPES, pH 7.3 containing 0.5 mg/mL Pefabloc (ROCHE DIAGNOSTICS, Mannheim,

Germany), 1x Complete Protease Inhibitor Cocktail (Roche), 3.5 μg/mL pepstatin (ROCHE), 20 mM benzamidine (SIGMA, St. Louis MO), 5 mM EDTA, and 5 mM EGTA. Breakage was performed at 4° C using a vortex mixer for 15 minutes. Cellular debris and beads were collected by centrifugation in a microcentrifuge and the supernatant was transferred to a second microcentrifuge tube that was again centrifuged to remove debris. Aliquots were removed and assayed for total protein using the BCA assay (PIERCE, Rockford, Illinois). Four μg of each lysate were then loaded on a 4-12 % NUPAGE BIS TRIS gel and subjected to electrophoresis in MOPS SDS running buffer (INVITROGEN, Carlsbad, CA). The gel was transferred to PVDF membrane (INVITROGEN , Carlsbad, CA) for Western blotting using MAb 86613M (BIODESIGN INTERNATIONAL, Saco, ME) as primary antibody and goat anti-mouse-IgG peroxidase conjugate (PERKIN ELMER, Boston, MA) as secondary antibody. Detection was performed using CN/DAB peroxidase substrate (PIERCE, Rockford, Illinois). Purified recombinant E. coli PA83 (see Examples 2 and 4) was used as standard.

The results are shown in FIG. 13. Transformant 9-1 of S. cerevisiae strain 1260 (lane 1) was one of the best producers of internally-produced rPA83 and was chosen for large-scale fermentation.

EXAMPLE 10:

Intracellular Expression of PA83 with Intact Furin Cleavage Site (cleavable PA83) in S. cerevisiae

The DNA encoding the PA83 protein with a mutated Furin cleavage site (Example 6) and with codons optimized for yeast expression was subcloned into pCR4-TOPO (INVITROGEN, Carlsbad, CA). The PA83 coding sequence was altered to encode an arginine at amino acid 167 of the cleavage site using the QUICKCHANGE Site-directed mutagenesis kit (STRATAGENE, La Jolla, CA). The primers used for mutating the site were:

Sense 5' CAACTCCAGAAAGAAGAGATCCACCTCCGCTGGTC - SEQ ID NO: 64 and antisense 5' GACCAGCGGAGGTGGATCTCTTCTTTCTGGAGTTG - SEQ ID NO: 65

The sequence of the modified plasmid was verified by DNA sequencing. The DNA for the cleavable construct was amplified from pCR4-TOPO as a *Bam*HI fragment using the sense primer described in the previous example,

5'CGCGGATCCCACAAAACAAAATGGAAGTTAAGCAAGAAAACAGA (SEQ ID NO: 63), and the antisense primer described in Examples 1 and 2.

5'CGCGGATCCTTAACCAATTTCGTAACCCTTCTTAGAGAA3' (SEQ ID NO: 29) The *Bam*HI fragment was subcloned in the proper orientation into a yeast expression vector (pGAL110, FIG. 4) to construct pPA83clGAL10#6. The sequence of the insert was verified by DNA sequencing. The plasmid was used to transform S. cerevisiae strain 1260 to leucine prototrophy as described in Examples 2 and 4. Production of cleavable PA83 was evaluated from several transformants of strain 1260 and uncleavable PA83 was produced from transformant 9-1 in small-scale fermentations conducted in YEHDG medium as described in Examples 2 and 4. Cell-pellets corresponding to 10 OD_{600} units were broken in the

presence of 0.5 g of 0.45 mm glass beads and 0.5 mL of breaking buffer (20 mM HEPES, pH 8.2, 5 mM EDTA, 1x "Complete" protease inhibitor cocktail (ROCHE DIAGNOSTICS, Mannheim, Germany) by continuous vortexing at high speed at 4° C for 10 minutes. Cellular debris and glass beads were collected by centrifugation in a microcentrifuge at 4° C for 10 minutes. The clarified supernatants were transferred to new tubes and assayed for total protein using the BCA assay (Pierce, Rockford, Illinois). Two and one half μg of each sample was loaded onto a 4-12 % NUPAGE BIS TRIS gel and run in MOPS SDS running buffer (INVITROGEN, Carlsbad, CA). The gel was transferred to PVDF membrane (INVITROGEN, Carlsbad, CA) and Western blotted using Mab 86613M (BIODESIGN, Saco, ME) as primary antibody and goat anti-mouse-IgG peroxidase conjugate (PERKIN ELMER, Boston, MA) as secondary antibody. Detection was performed using CN/DAB peroxidase substrate (PIERCE, Rockford, IL). Purified recombinant E. coli PA83 (see Examples 2 and 4) was used as standard. No detection was obtained with a cell lysate containing the vector control. Cleavable PA83 was detected in all three yeast transformants tested and co-migrated with uncleavable PA83 produced in S. cerevisiae. Transformant 5C was chosen as one of the best producers of cleavable PA83.

Subsequently, production of uncleavable PA83 by yeast strain 9-1 and cleavable PA83 produced by strain 5-C, fermented in shake flasks in YEHDG medium, was assessed. Strain 5-C was also fermented in YEHDG in a culture tube. The tube fermentations were conducted as described in Examples 2 and 4. The shake-flask fermentations were conducted as described in Example 2, except that the galactose induction was conducted in 50 mL YEHDG medium in a 250-mL flask with inocula scaled-up proportionately. Cell lysates were prepared and evaluated by Western blot as described in Example 2. The results are shown in FIG. 14. Purified recombinant E. coli PA83 (lane 1) was used as standard. The additional protein detected below rPA83 is a degradation product that is sometimes observed. No detection was obtained with a cell lysate containing the vector control (lane 2). Production of uncleavable PA83 was greater than cleavable PA83 (compares lanes 3 and 4 to 5 and 6). Production of cleavable PA83 was greater in culture tube (5-mL volume) than in a shake flask (compare lanes 7 and 8 to 5 and 6).

EXAMPLE 11:

Fermentation and Purification of Internally-Expressed PA63

Frozen seed stock of yeast strain 8-3 (strain 1260 transformed with plasmid p63GAL10#2) was used for large-scale fermentation and purification. The vial of seed stock was thawed and 1.0 mL was used to inoculate a 250-mL Erlenmeyer flask containing 50 mL of a leucine-free selective medium (5X Leu⁻ medium. Bayne et al., 1988.) containing 4 % dextrose. The flask was incubated at 28° C, 250 rpm on a rotary shaker. After 24 hours (residual glucose 22.3 g/L), a culture volume of 11 mL was added to a 2-L flask containing 877 mL of the same medium. Again, the flask was incubated at 28° C and agitated at 250 rpm. After 24.5 hours (residual glucose 12.9 g/L), the contents of the 2-L flask were used to

inoculate a 20-L reactor containing a chemically defined medium (Oura, E., 1974) optimized for our strains. This medium contained 20 g/L glucose followed by 40 g/L galactose for induction. The reactor was operated at 28° C, 4.7 L/min, 15 psig, and 300 rpm. Under these conditions, dissolved oxygen levels were maintained at greater than 30 % of saturation. Cellular growth was monitored by glucose consumption, optical density (A600 nm, 1-cm cuvettes), dry cell weight, galactose utilization and ethanol production. Cultivation continued for 90 h reaching an OD_{600} of 35 and a dry cell weight of 19.7 g/L.

The culture was harvested via hollow fiber tangential flow filtration (AMICON H5MP01-43 cartridge) using an AMICON DC-10 harvest skid (MILLIPORE, Billerica, MA). The permeate was discarded and the cells were concentrated down to 1.35 L. Cells were then diafiltered with PBS, and collected by centrifugation at 8000 rpm, 4° C for 20 minutes using a Sorvall Evolution RC (SLA-3000 rotor). Cells were stored at –70° C.

To evaluate production of PA63 by strain 8-3 in large-scale fermentation, cell lysates were prepared from 10 OD_{600} units of the harvested culture and evaluated as detailed in Examples 2 and 3. Production of PA63 from a small-scale culture tube fermentation (72 h, complex YEHDG medium) was also assessed. The results of a Western blot are shown in FIG. 15. It can be seen that the protein produced from the large-scale fermentation, lanes 5 and 6, comigrated with PA63 produced in a tube fermentation, lanes 7, and 8 and with the control PA63 shown in lanes 1, 2, 11 and 12. It is apparent that the yield of PA63 from the large-scale fermentation is greater than that of the tube fermentation. The titer of PA63 from the large-scale fermentation was estimated to be 220 μg per mL and the % total protein, 12 %, compared to 65 μg/mL and 8.0 % of the total protein for the tube fermentation (using the semi-quantitative Western method described in Example 3).

The quantitation of expression in the large-scale fermentation was confirmed by ELISA as described in Example 3. Volumetric titers of 360 and 80 μg per mL were determined for the large-scale and tube fermentations, respectively. The enhanced yields from the large-scale fermentation can probably be attributed to both decoupling of cell growth from induction of PA63 expression, and selective pressure for plasmid maintenance.

The purification process for PA63 from yeast is presented in FIG. 16. Frozen yeast cells were thawed in a cold-water bath for several hours. The cells were resuspended in five times the volume of cold breaking buffer (0.2 M HEPES, pH 7.4, 2 mM $MgCl_2$, 0.5 mg/mL PEFABLOC (Roche Applied Science, Indianapolis, IN), 1x "complete" EDTA free protease inhibitor mix (ROCHE APPLIED SCIENCE, Indianapolis, IN), 3.5 μg/mL pepstatin (ROCHE APPLIED SCIENCE, Indianapolis, IN), 20 mM benzamidine (SIGMA, St. Louis, MO), and 1 μL benzonase (EM INDUSTRIES, Hawthorne, NY) per gram of cells.

The yeast cells were ruptured by 4 sequential passes through a microfluidizer apparatus (Model 110S, MICROFLUIDICS, Newton, MA) at a pressure of 18,000 psi. The temperature of the lysate was

maintained at <15° C throughout the procedure by cooling between passes in an ice water bath. The pH of the lysate remained at 7.4 throughout the procedure.

Following the cell breakage, EDTA and EGTA were added to the lysate at final concentrations of 5 mM each. Insoluble material was removed from the lysate by low speed centrifugation (10,000 x g) in a Sorvall RC-5C refrigerated centrifuge with a GS3 rotor for 20 minutes at 4° C.

The supernatant of the low speed spin was clarified by filtration through a series of 47 mm diameter filters (AP15, AP25, and 5 μM (DURAPORE White Hydrophilic Disc SVLP, MILLIPORE, Billerica, MA). The clarified lysate was divided into aliquots and stored at –70° C.

Preliminary attempts were made to purify PA63 under non-denaturing conditions using a variety of column chemistries. None of the chemistries were able to resolve a single defined PA63 peak. In all cases investigated, the PA63 bled slowly off of the columns across the entire elution gradient. We believe this may be due in part to the active nature of PA63, which is designed to self-assemble into heptamers. This activity may be responsible for the formation of multimeric PA63 structures or PA63-yeast protein aggregates in the lysate, which prevents isolation of a single enriched PA63 fraction under non-denaturing conditions.

Therefore, further processing of the lysate was performed under denaturing conditions because the PA63 was not amenable to purification under non-denaturing conditions. Non-ionic denaturants are preferred because ionic denaturants would not be compatible with the chromatography resins employed in subsequent steps. Specifically, the lysate was made 6 M in urea and was applied to a 1 L POROS 50HS column (APPLIED BIOSYSTEMS, Foster City, CA) equilibrated in 6 M urea/20 mM HEPES, pH 7.3 at a flow rate of 35 mL/min using a WATERS 650 Prep system with a Linear UV/VIS detector (WATERS, Milford, MA). Proteins were eluted from the column using a 0-1 M NaCl gradient in 6 M urea/HEPES buffer.

Total protein in column fractions was determined using a commercial bicinchoninic acid (BCA) assay (PIERCE CHEMICAL, Rockford, IL) and SDS-PAGE with Western blotting. The PA63-containing fractions were pooled and concentrated over a 10-kDa tangential flow membrane (PREPSCALE TFF, MILLIPORE, Billerica, MA). The concentrated pool was diluted with 6 M urea-containing 20 mM HEPES buffer and then applied to a 50 mL SOURCE Q (AMERSHAM BIOSCIENCES, Piscataway, NJ) anion exchange column equilibrated in 6 M urea/HEPES and again purified with a 0-1 M NaCl gradient in the presence of 6 M urea. The PA63 containing fractions were pooled and dialyzed extensively against 20 mM HEPES, pH 7.3 containing 0.15 M NaCl and 16 μM $CaCl_2$. The product of the Source Q step was >90 % pure PA63 by SDS-PAGE analysis. The pooled product was filtered at 0.2 μm and stored in aliquots at –70° C.

FIG. 17 presents the results of SDS-PAGE analysis by Coomassie staining and Western blotting for the final product and intermediate process retains. Table 2 summarizes the material balance of protein through the process.

Table 2. Material balance and protein recovery at research-scale

Process Retain	Volume (mL)	Protein Concentration (μg/mL)	Total Protein (mg)
Clarified Lysate	320	13200	4224
50HS Pool	420	1000	420
Source Q Pool (product)	240	589	141

EXAMPLE 12:

Immunogenicity of Recombinant PA63

An aspect of this invention indicates that the purified yeast recombinant PA63 protein (purified as described in Example 11) can induce in mammals a robust immune response that is cross-reactive to PA83 (the precursor form of PA63). Mice (five per group) were vaccinated on days 0, 7 and 21 with antigens formulated on Merck aluminum adjuvant (MAA), *E. coli* recombinant PA83, or yeast recombinant PA63. One group of mice received MAA alone. Mice were bled on day 29 to measure antibody titers to recombinant antigens (Table 3). Titers were measured by coating 96 well plates with 0.1 μg/well of either rPA63 or rPA83. The secondary antibody was goat anti-mouse IgG-HRP (SOUTHERN BIOTECHNOLOGY, Birmingham, AL) used at 1:6000 dilution. The plate was developed with TMB (PIERCE CHEMICALS, Rockford, IL) and read at OD_{450}.

Table 3. Mouse end point titers to recombinant proteins, GMT of five mice per group

Group	GMT to rPA63	GMT to rPA83
50 μg rPA83	1,350,519	3,103,372
50 μg rPA63	588,128	588,128
MAA	<100	<100

Rabbits (ten per group) were vaccinated on days 0, 1 month, 2 months, and 3 months with recombinant yeast PA63 formulated on MAA. One group of rabbits received adjuvant alone, and one group of five rabbits received two 125 μL doses of the currently licensed vaccine Anthrax Vaccine Adsorbed (AVA) (BIOPORT CORP., Lansing, MI). All rabbits were bled at 2 weeks post last vaccination to measure antibody titers to recombinant PA83 (Table 4). Titers were measured as described above but the secondary antibody was goat anti-rabbit IgG-HRP (SOUTHERN BIOTECHNOLOGY, Birmingham, AL) used at 1:6000.

Table 4. Rabbit end point titers to recombinant PA83, GMT of ten rabbits per group, except for the AVA group in which there were five rabbits

Group	GMT to rPA83
0.5 μg rPA63	166,491
5.0 μg rPA63	455,795
50 μg rPA63	649,507
AVA	799,882
MAA	<100

Monkeys (three per group) were vaccinated on days 0, 2 months, and 6 months with recombinant yeast PA63 formulated on MAA. One group of monkeys received adjuvant alone, and one group of monkeys received three human doses (500 μL) of the currently licensed AVA vaccine. Monkeys were bled at two weeks post dose three to measure antibody titers to recombinant PA83 (Table 5). End point titers were measured as described above except that the secondary antibody was goat anti-monkey IgG-HRP (SOUTHERN BIOTECHNOLOGY, Birmingham, AL) used at 1:5000 dilution.

Table 5. Monkey end point titers to recombinant PA83, GMT of three monkeys per group

Group	GMT to rPA83
5 μg rPA63	63,496
50 μg rPA63	100,793
AVA	100,793
MAA	1,000

EXAMPLE 13:

Protection against exposure to B. anthracis by yeast recombinant PA63

The immune response induced by the vaccine formulated with recombinant PA63 produced in yeast confers protection against a lethal aerosol exposure of the deadly B. anthracis. Rabbits were vaccinated as described above and exposed to a lethal aerosol challenge of B. anthracis Ames strain. Spores of the B. anthracis Ames strain were grown in Leighton and Doi medium (Leighton, T. J. and Doi, R. H., 1971), purified by centrifugation through 58% HYPAQUE-76 (NYCODED, INC., Princeton, NJ) and resuspended in sterile water containing 1% phenol, and stored at 2-8° C until used. Immediately before aerosol challenge, the spores were diluted in sterile water for injection to 2.2-2.8 X 10^9 CFU/ml and heat-shocked at 60° C for 45 min. After respiratory minute volumes were measured, animals were exposed in a head-only chamber to a spore aerosol generated by a three-jet collision

nebulizer. For each animal, the concentration of spores in the aerosol inhaled dose (expressed as LD_{50}) was determined by plating a sample from an all glass impinger onto tryptic soy agar plates (DIFCO, Detroit, MI). Animals were observed for a period of two weeks and deaths from anthrax recorded (Table 6).

Table 6. Rabbit survival data after lethal challenge of *B. anthracis* Ames strain

Group	% Mortality	Spore Dose (LD_{50})
0.5 μg rPA63, 10 animals	50	733
5 μg rPA63, 9 animals	11	673
50 μg rPA63, 10 animals	40	611
AVA, 5 animals	0	591
MAA, 10 animals	100	624

Monkeys were vaccinated as described above, and given a booster dose of either 5 μg or 50 μg or MAA alone, and exposed to a lethal aerosol challenge of B. anthracis Ames strain, as described above, two weeks after the booster injection. The challenge dose was approximately 1000 LD_{50} per animal. Animals were observed for a period of four weeks and deaths from anthrax recorded (Table 7).

Table 7. Monkey survival data after lethal challenge of *B. anthracis* Ames strain

Group (groups of 3)	% Mortality
5 μg rPA63	0
50 μg rPA63	30
AVA	30
MAA	100

The results from these single monkey and rabbit protection studies indicate that yeast-expressed rPA63 is a very promising candidate for a component of an anthrax vaccine. The data also suggest that rPA63 has the potential to be as effective as AVA, the current vaccine.

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WHAT IS CLAIMED IS:

1. A method for the production of recombinant Bacillus anthracis Protective Antigen protein, comprising:

a) providing an expression vector including a polynucleotide comprising a nucleotide sequence encoding a Bacillus anthracis Protective Antigen protein, said nucleotide sequence being codon-optimized for expression in yeast,

b) transforming a yeast with the vector,

c) fermenting the transformed yeast under conditions appropriate for the expression of the Protective Antigen protein, and

d) isolating the Protective Antigen protein.

2. The method according to Claim 1 wherein the Protective Antigen protein is PA63.

3. The method according to Claim 1 wherein the Protective Antigen protein is PA83.

4. The method according to Claim wherein the Protective Antigen protein encompasses a portion of the protein that, upon introduction into a patient, stimulates an immunoprotective response against Bacillus anthracis.

5. The method according to Claim 1 wherein the yeast is selected from the group consisting of Pichia pastoris, Hansenula sp., Candidia sp., Saccharomyces cerevisiae, Saccharomyces carlsbergensis and Kluyveromyces lactis.

6. The process according to Claim 1 wherein the yeast host comprises at least one genetic alteration selected from the group comprising alterations to prevent proteolysis, improve removal of the secretory leader and to promote proper glycosylation.

7. The process according to Claim 2 wherein the PA83 protein is modified to prevent protease cleavage of the PA83 at the cleavage site recognized by furin.

8. A synthetic polynucleotide comprising a sequence encoding a Bacillus anthracis Protective Antigen protein, the amino acid sequence of said Protective Antigen protein maintaining wild-type Protective Antigen protein immunogenicity, wherein said polynucleotide sequence encoding the Protective Antigen protein is comprised of codons optimal for expression in a yeast host.

9. A polynucleotide according to Claim 8 wherein the protein is selected from the group consisting of a PA63 protein having a wild type amino acid sequence, a PA83 protein having a wild type amino acid sequence and a PA83 protein modified to prevent protease cleavage of the PA83 at the cleavage site recognized by furin and/or other mammalian processing enzymes.

10. A polynucleotide according to Claim 8 which is DNA.

11. A polynucleotide according to Claim 8 which comprises a sequence selected from the group consisting of SEQ ID NOs: 49, 51, 55 and 57.

12. A yeast expression vector comprising a polynucleotide according to Claim 8.

13. The vector of Claim 12 wherein the polynucleotide is operably associated with at least one regulatory sequence recognized by a yeast host cell.

14. The vector according to Claim 13 wherein the regulatory sequences comprise a GAL10 promoter and a yeast transcriptional terminator.

15. The vector according to Claim 13 wherein the regulatory sequences comprise a GAL1 promoter and a yeast transcriptional terminator.

16. A yeast host cell harboring a vector according to Claim 12.

17. A yeast host cell harboring a vector according selected from the group consisting of the vectors of Claim 14 and Claim 15.

18. A vaccine comprising a recombinant Bacillus anthracis Protective Antigen protein wherein said Protective Antigen protein was produced in yeast by expression of a yeast codon-optimized nucleotide sequence.

19. A method of vaccination of a patient against Bacillus anthracis comprising administering of a vaccine of Claim 18 to the patient.

20. The method of Claim 19 wherein the patient is a human.

21. The method of Claim 19 wherein the patient is selected from the group consisting of domesticated animals and livestock.

22. The method of Claim 19 wherein the vaccine is administered by injection.

23. The method of Claim 1 wherein the Protective Antigen protein is secreted from the yeast.

24. The method of Claim 1 wherein the Protective Antigen protein is isolated from the cytoplasm of the yeast.

25. The expression vector of Claim 12 wherein a polynucleotide encoding a secretion signal recognized by a yeast is operably linked to the polynucleotide encoding the Protective Antigen protein.

26. A process of isolating the 63 kilodalton form of Protective Antigen from a culture of yeast cells expressing a recombinant PA63 gene comprising,

disrupting the yeast cells to create a suspension and clarifying the suspension,

adding a denaturant to the suspension,

applying the suspension to a column containing a cation exchange resin in a denaturing buffer,

eluting the PA63 from the cation exchange resin with a salt gradient in the denaturing buffer,

applying the PA63 to a column containing an anion exchange resin in a denaturing buffer,

eluting the PA63 from the anion exchange resin with a salt gradient in the denaturing buffer, and

dialyzing the PA63 to remove the denaturant.

27. The process of Claim 26 wherein the denaturant and denaturing buffer contains a sufficient amount of urea to denature the PA63.

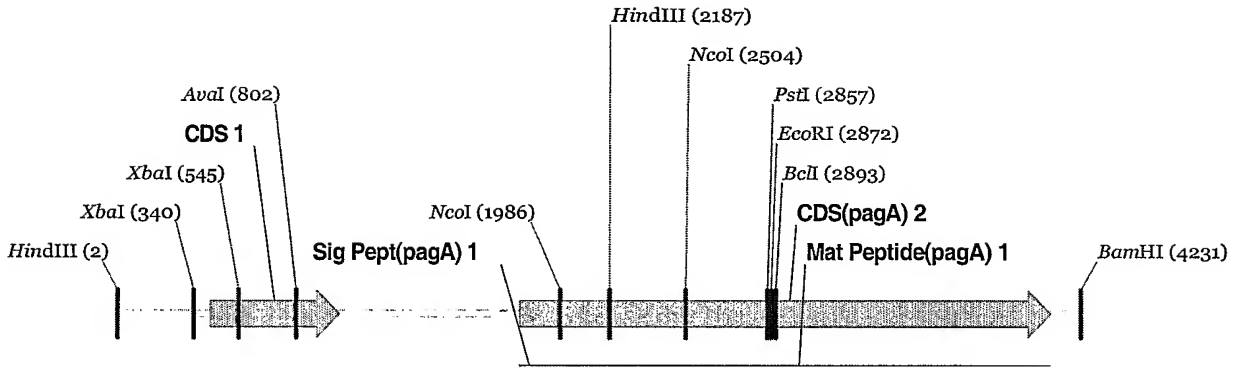

FIGURE 1A

BACPAG

4235 bp

Bacterial sequence (PA63)

agtacaagtgctggacctacggttccagaccgtgacaatgatggaatccctga
ttcattagaggtagaaggatatacggttgatgtcaaaaataaaagaacttttc
tttcaccatggatttctaatattcatgaaaagaaaggattaaccaaatataaa
tcatctcctgaaaaatggagcacggcttctgatccgtacagtgatttcgaaaa
ggttacaggacggattgataagaatgtatcaccagaggcaagacacccccttg
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gttgaatatttctagtttacggcaagatggaaaaacatttatagattttaaaa
aatataatgataaattaccgttatatataagtaatcccaattataaggtaaat
gtatatgctgttactaaagaaaacactattattaatcctagtgagaatgggga
tactagtaccaacgggatcaagaaaatttttaatctttttctaaaaaaggctatg
agatagga

Translation

STSAGPTVPDRDNDGIPDSLEVEGYTVDVKNKRTFLSPWISNIHEKKGLTKYK
SSPEKWSTASDPYSDFEKVTGRIDKNVSPEARHPLVAAYPIVHVDMENIILSK
NEDQSTQNTDSETRTISKNTSTSRTHTSEVHGNAEVHASFFDIGGSVSAGFSN
FIGSNSSTVAIDHSLSLAGERTWAETMGLNTADTARLNANIRYVNTGTAPIYN
VLPTTSLVLGKNQTLATIKAKENQLSQILAPNNYYPSKNLAPIALNAQDDFSS
TPITMNYNQFLELEKTKQLRLDTDQVYGNIATYNFENGRVRVDTGSNWSEVLP
QIQETTARIIFNGKDLNLVERRIAAVNPSDPLETTKPDMTLKEALKIAFGFNE
PNGNLQYQGKDITEFDFNFDQQTSQNIKNQLAELNATNIYTVLDKIKLNAKMN
ILIRDKRFHYDRNNIAVGADESVVKEAHREVINSSTEGLLLNIDKDIRKILSG
YIVEIEDTEGLKEVINDRYDMLNISSLRQDGKTFIDFKKYNDKLPLYISNPNY
KVNVYAVTKENTIINPSENGDTSTNGIKKILIFSKKGYEIG

FIGURE 1B

PA63 Yeast codon optimized

TCCACCTCCGCTGGTCCAACTGTTCCAGACAGAGACAACGACGGTATTCCAGA
CTCCTTGGAAGTCGAAGGTTACACCGTCGACGTCAAGAACAAGAGAACCTTCT
TGTCTCCATGGATCTCCAACATTCACGAAAAGAAGGGTTTGACCAAGTACAAG
TCTTCCCCAGAAAAGTGGTCTACTGCTTCCGACCCATACTCCGACTTCGAAAA
GGTCACCGGTAGAATCGACAAGAACGTCTCCCCAGAAGCTAGACACCCATTGG
TTGCTGCTTACCCAATTGTCCACGTTGACATGGAAAACATCATTTTGTCCAAG
AACGAAGACCAATCCACCCAAAACACGGACTCCGAAACCAGAACTATCTCTAA
GAACACCTCCACTTCTAGAACCCACACTTCCGAAGTCCACGGTAACGCTGAAG
TTCACGCTTCTTTCTTCGACATTGGTGGTTCCGTCTCTGCTGGTTTCTCCAAC
TCCAACTCCTCCACCGTCGCTATCGACCACTCTTTGTCCTTGGCTGGTGAAAG
AACTTGGGCTGAAACCATGGGTTTGAACACTGCTGACACCGCTAGATTGAACG
CTAACATTAGATACGTCAACACTGGTACCGCTCCAATCTACAACGTCTTGCCA
ACCACCTCCTTGGTCTTGGGTAAGAACCAAACCTTGGCTACTATCAAGGCTAA
GGAAAACCAATTGTCCCAAATCTTGGCTCCAAACAACTACTACCCATCCAAGA
ACTTGGCTCCAATCGCTTTGAACGCTCAAGACGACTTCTCCTCTACTCCAATC
ACCATGAACTACAACCAATTCTTGGAATTGGAAAAGACTAAGCAATTGCGTTT
GGACACCGACCAAGTTTACGGTAACATTGCTACTTACAACTTCGAAAACGGTA
GAGTCAGAGTCGACACCGGTTCCAACTGGTCTGAAGTCTTGCCACAAATCCAA
GAAACCACCGCTAGAATCATCTTCAACGGTAAGGACTTGAACTTGGTTGAAAG
AAGAATTGCTGCTGTCAACCCATCCGACCCATTGGAAACCACCAAGCCAGACA
TGACCTTGAAGGAAGCTTTGAAGATCGCTTTCGGTTTCAACGAACCAAACGGT
AACTTGCAATACCAAGGTAAGGACATCACCGAATTCGACTTCAACTTCGACCA
ACAAACCTCTCAAAACATCAAGAACCAATTGGCTGAATTGAACGCTACCAACA
TTTACACTGTTTTGGACAAGATCAAGTTGAACGCCAAGATGAACATCTTGATC
AGAGACAAGAGATTCCACTACGACAGAAACAACATTGCTGTCGGTGCTGACGA
ATCCGTTGTCAAGGAAGCCCACAGAGAAGTCATCAACTCCTCCACCGAAGGTT
TGTTGTTGAACATCGACAAGGACATCAGAAAGATCTTGTCCGGTTACATCGTC
GAAATTGAAGACACCGAAGGTTTGAAGGAAGTCATCAACGACAGATACGACAT
GTTGAACATTTCCTCTTTGAGACAAGACGGTAAGACCTTCATCGACTTCAAGA
AGTACAACGACAAGTTGCCATTGTACATTTCCAACCCAAACTACAAGGTCAAC
GTTTACGCTGTCACTAAGGAAAACACCATCATCAACCCATCCGAAAACGGTGA
CACTTCCACCAACGGTATCAAGAAGATTTTGATCTTCTCTAAGAAGGGTTACG
AAATTGGT

Translation

STSAGPTVPDRDNDGIPDSLEVEGYTVDVKNKRTFLSPWISNIHEKKGLTKYK
SSPEKWSTASDPYSDFEKVTGRIDKNVSPEARHPLVAAYPIVHVDMENIILSK
NEDQSTQNTDSETRTISKNTSTSRTHTSEVHGNAEVHASFFDIGGSVSAGFSN
SNSSTVAIDHSLSLAGERTWAETMGLNTADTARLNANIRYVNTGTAPIYNVLP
TTSLVLGKNQTLATIKAKENQLSQILAPNNYYPSKNLAPIALNAQDDFSSTPI
TMNYNQFLELEKTKQLRLDTDQVYGNIATYNFENGRVRVDTGSNWSEVLPQIQ
ETTARIIFNGKDLNLVERRIAAVNPSDPLETTKPDMTLKEALKIAFGFNEPNG
NLQYQGKDITEFDFNFDQQTSQNIKNQLAELNATNIYTVLDKIKLNAKMNILI
RDKRFHYDRNNIAVGADESVVKEAHREVINSSTEGLLLNIDKDIRKILSGYIV
EIEDTEGLKEVINDRYDMLNISSLRQDGKTFIDFKKYNDKLPLYISNPNYKVN
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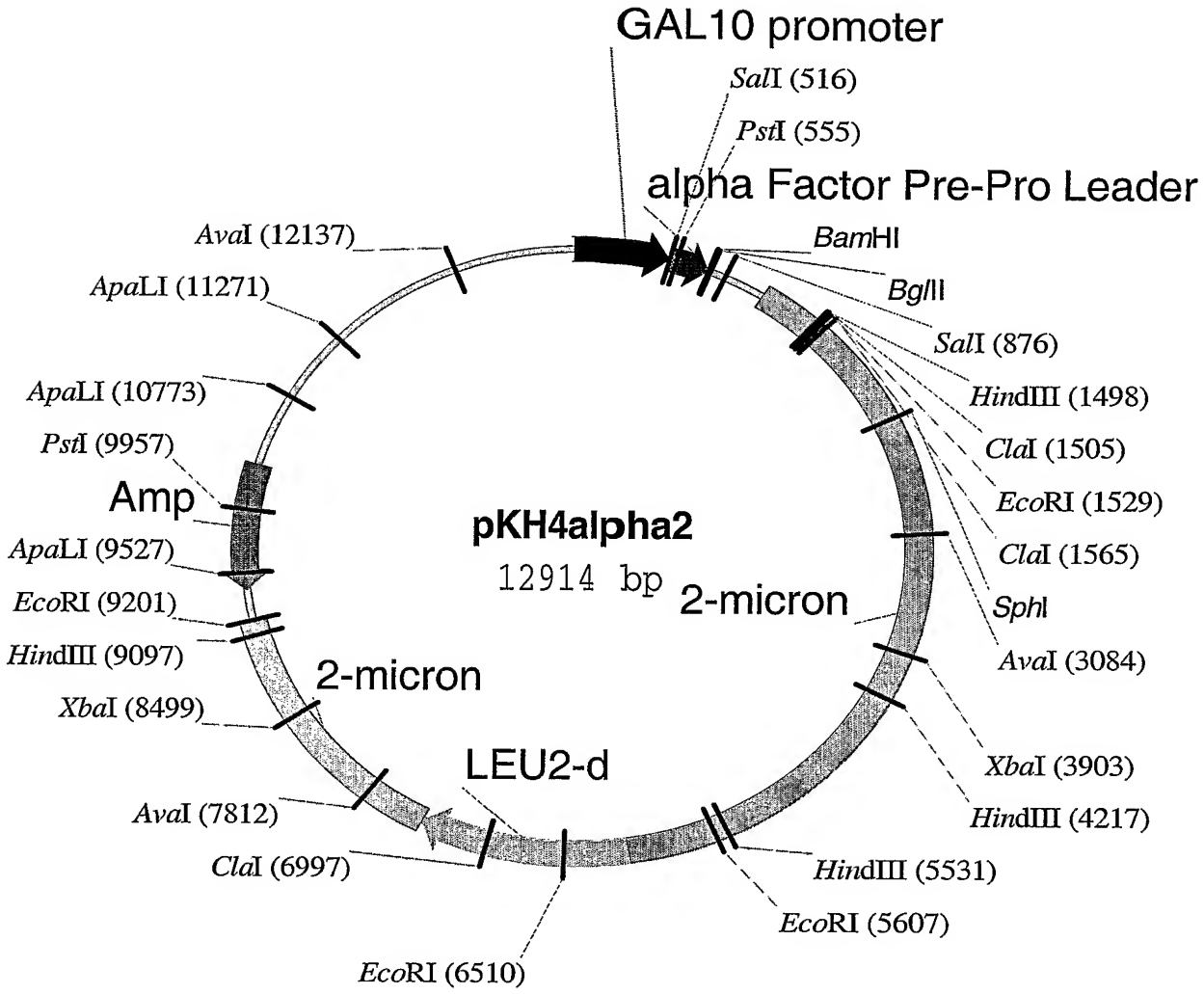
FIGURE 2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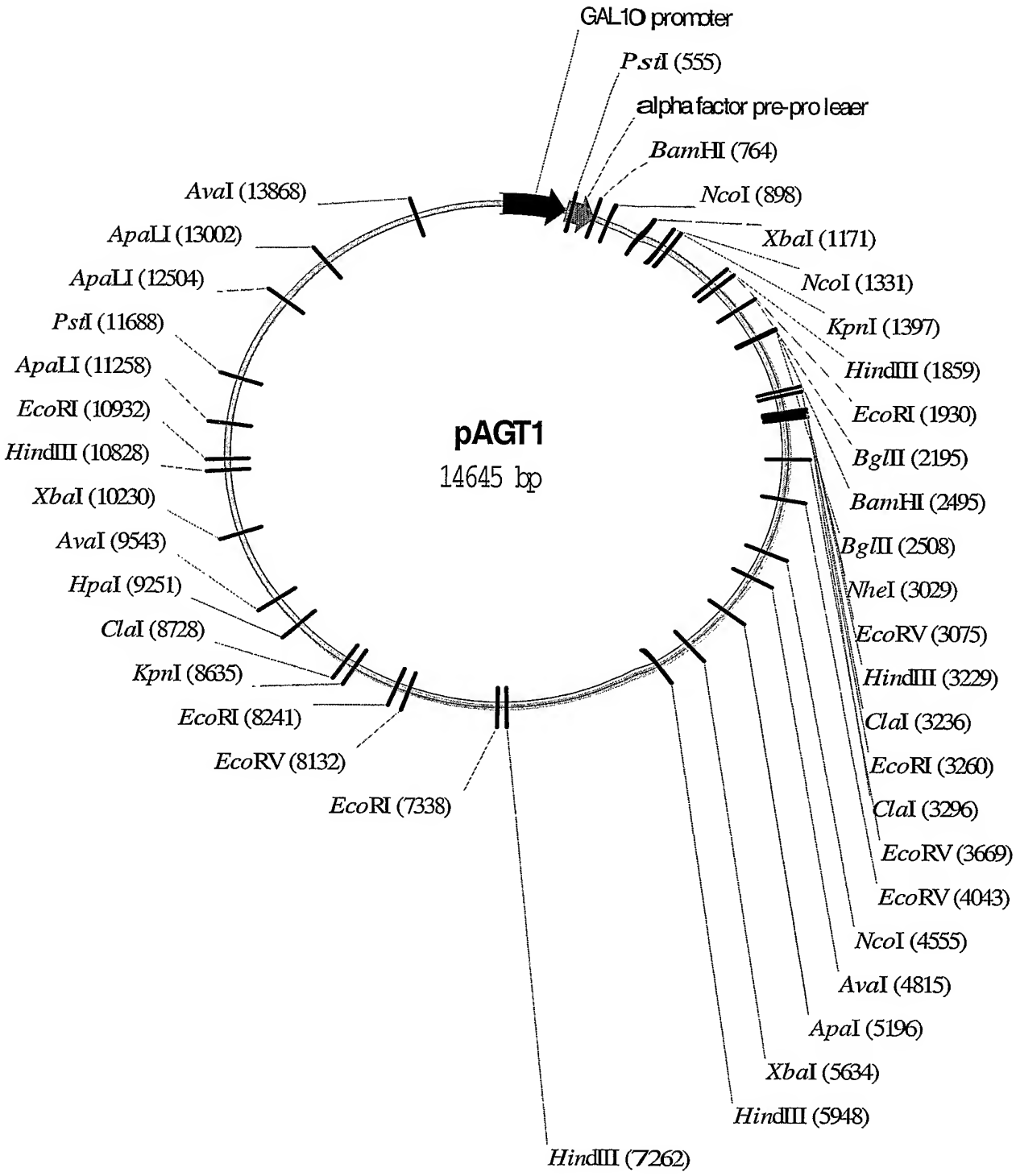
SalI (516)
PstI (555)
alpha Factor Pre-Pro Leader
BamHI
BglII
SalI (876)
HindIII (1498)
ClaI (1505)
EcoRI (1529)
ClaI (1565)
SphI
AvaI (3084)
XbaI (3903)
HindIII (4217)
HindIII (5531)
EcoRI (5607)
EcoRI (6510)
ClaI (6997)
AvaI (7812)
XbaI (8499)
HindIII (9097)
EcoRI (9201)
ApaLI (9527)
Amp
PstI (9957)
ApaLI (10773)
ApaLI (11271)
AvaI (12137)
pKH4alpha2
12914 bp
2-micron
2-micron
LEU2-d

FIGURE 3A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alpha factor pre-pro leaer
BamHI (764)
NcoI (898)
XbaI (1171)
NcoI (1331)
KpnI (1397)
HindIII (1859)
EcoRI (1930)
BglII (2195)
BamHI (2495)
BglII (2508)
NheI (3029)
EcoRV (3075)
HindIII (3229)
ClaI (3236)
EcoRI (3260)
ClaI (3296)
EcoRV (3669)
EcoRV (4043)
NcoI (4555)
AvaI (4815)
ApaI (5196)
XbaI (5634)
HindIII (5948)
HindIII (7262)
EcoRI (7338)
EcoRV (8132)
EcoRI (8241)
KpnI (8635)
ClaI (8728)
HpaI (9251)
AvaI (9543)
XbaI (10230)
HindIII (10828)
EcoRI (10932)
ApaLI (11258)
PstI (11688)
ApaLI (12504)
ApaLI (13002)
AvaI (13868)
pAGT1
14645 bp

FIGURE 3B

pGAL110

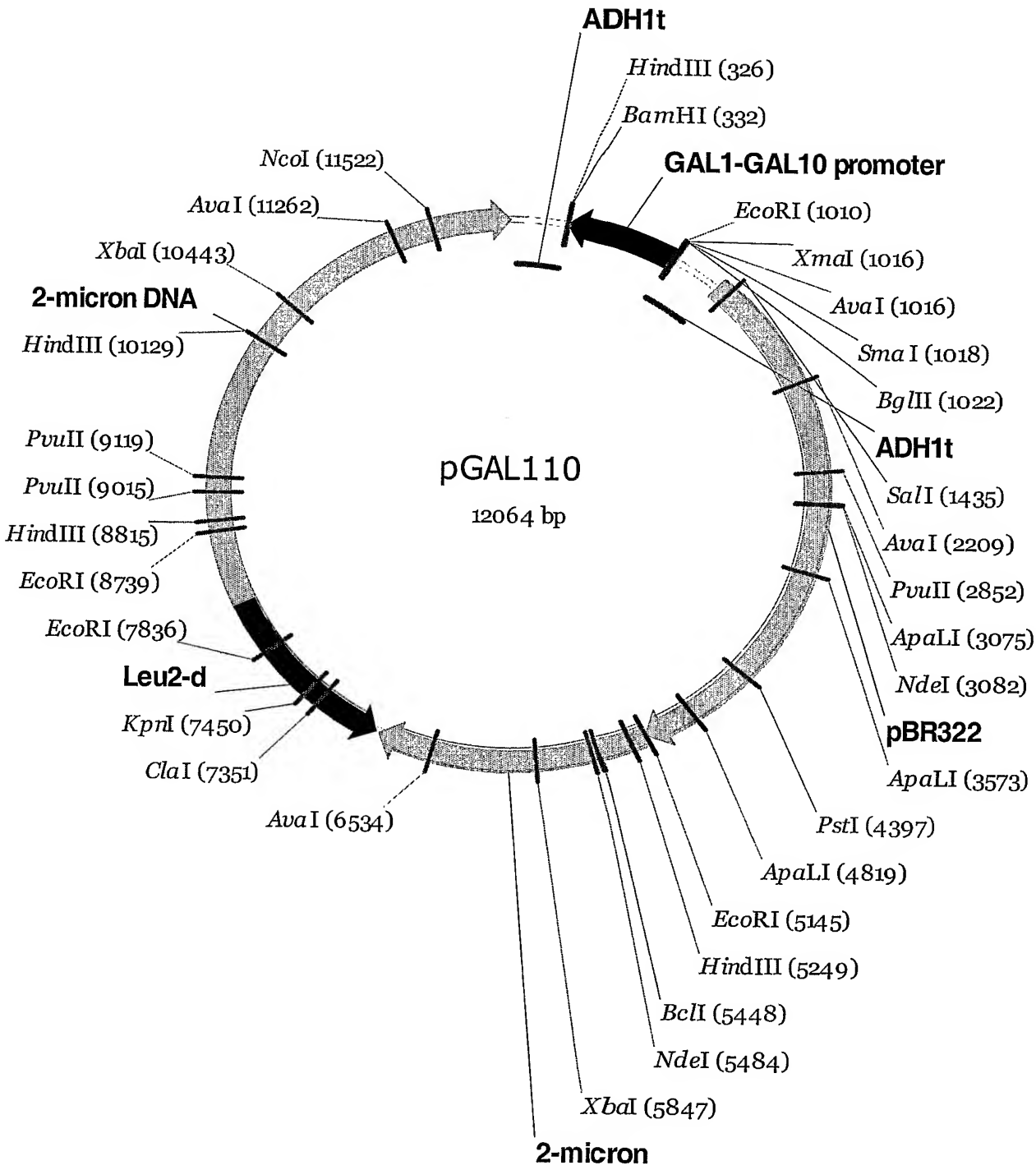

FIGURE 4

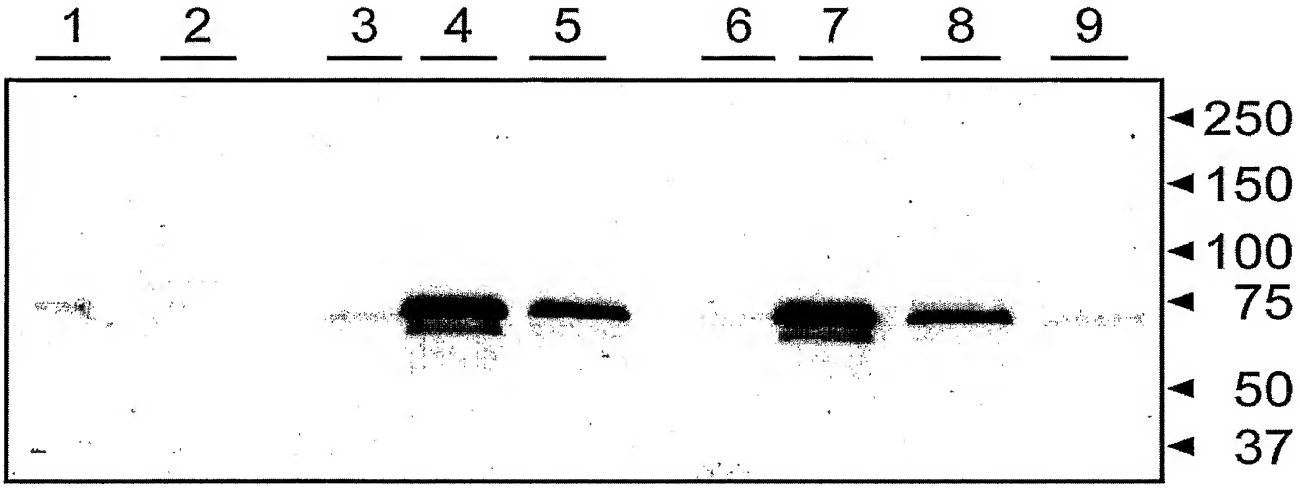

FIGURE 5

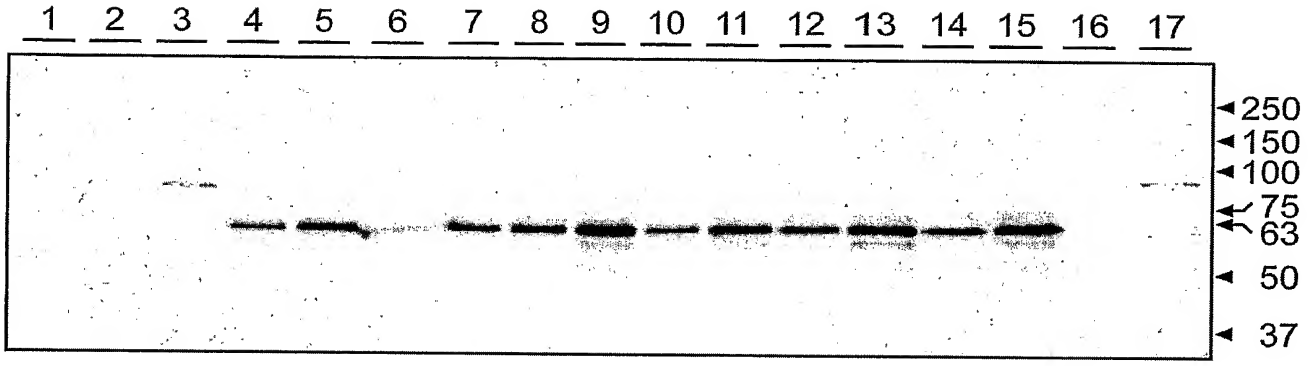

FIGURE 6

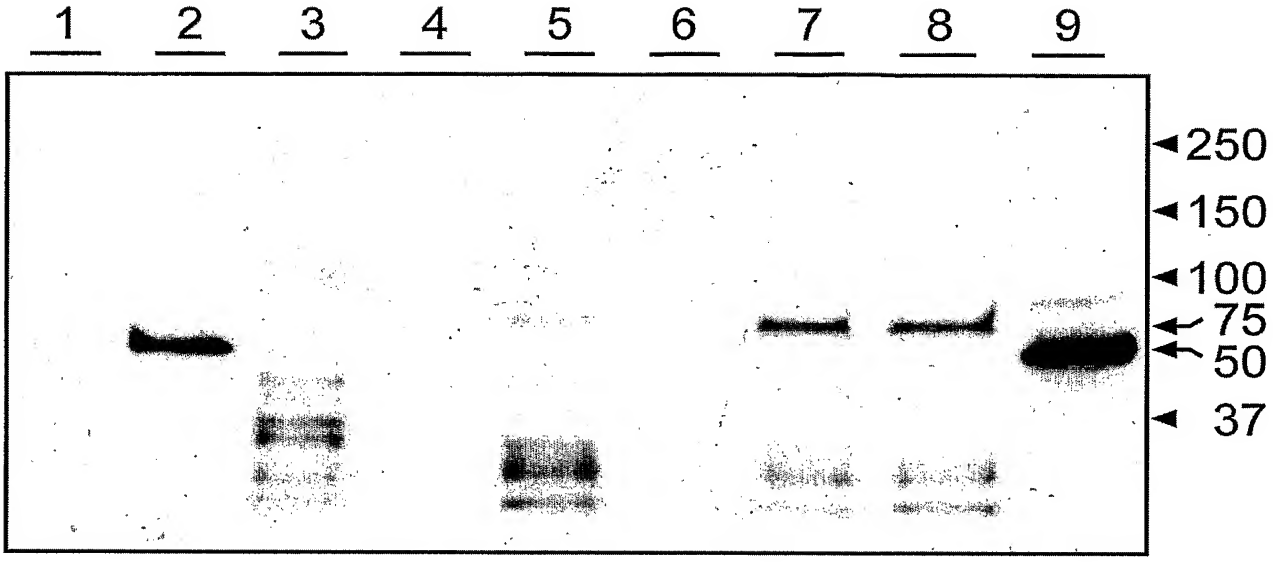

FIGURE 7

gaagttaaacaggagaaccggttattaaatgaatcagaatcaagttcccaggggttactag
gatactattttagtgatttgaattttcaagcacccatggtggttacctcttctactacagg
ggatttatctattcctagttctgagttagaaaatattccatcggaaaaccaatattttcaa
tctgctatttggtcaggatttatcaaagttaagaagagtgatgaatatacatttgctactt
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gaaaatcctactgaaaaaggattggatttcaagttgtactggaccgattctcaaaataaaa
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aagaaaaaagcgaagtacaagtgctggacctacggttccagaccgtgacaatgatggaatc
cctgattcattagaggtagaaggatatacggttgatgtcaaaaataaaagaacttttcttt
caccatggatttctaatattcatgaaaagaaaggattaaccaaatataaatcatctcctga
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tactgaagggcttaaagaagttataaatgacagatatgatatgttgaatatttctagttta
cggcaagatggaaaaacatttatagattttaaaaaatataatgataaattaccgttatata
taagtaatcccaattataaggtaaatgtatatgctgttactaaagaaaacactattattaa
tcctagtgagaatggggatactagtaccaacgggatcaagaaaatttttaatcttttctaaa
aaaggctatgagatagga

FIGURE 8A

Translation with Domain 4 underlined

```
  1    EVKQENRLLN ESESSSQGLL GYYFSDLNFQ APMVVTSSTT GDLSIPSSEL
 51    ENIPSENQYF QSAIWSGFIK VKKSDEYTFA TSADNHVTMW VDDQEVINKA
101    SNSNKIRLEK GRLYQIKIQY QRENPTEKGL DFKLYWTDSQ NKKEVISSDN
151    LQLPELKQKS SNSRKKRSTS AGPTVPDRDN DGIPDSLEVE GYTVDVKNKR
201    TFLSPWISNI HEKKGLTKYK SSPEKWSTAS DPYSDFEKVT GRIDKNVSPE
251    ARHPLVAAYP IVHVDMENII LSKNEDQSTQ NTDSETRTIS KNTSTSRTHT
301    SEVHGNAEVH ASFFDIGGSV SAGFSNSNSS TVAIDHSLSL AGERTWAETM
351    GLNTADTARL NANIRYVNTG TAPIYNVLPT TSLVLGKNQT LATIKAKENQ
401    LSQILAPNNY YPSKNLAPIA LNAQDDFSST PITMNYNQFL ELEKTKQLRL
451    DTDQVYGNIA TYNFENGRVR VDTGSNWSEV LPQIQETTAR IIFNGKDLNL
501    VERRIAAVNP SDPLETTKPD MTLKEALKIA FGFNEPNGNL QYQGKDITEF
551    DFNFDQQTSQ NIKNQLAELN ATNIYTVLDK IKLNAKMNIL IRDKRFHYDR
601    NNIAVGADES VVKEAHREVI NSSTEGLLLN IDKDIRKILS GYIVEIEDTE
651    GLKEVINDRY DMLNISSLRQ DGKTFIDFKK YNDKLPLYIS NPNYKVNVYA
701    VTKENTIINP SENGDTSTNG IKKILIFSKK GYEIG
```

FIGURE 8B

Domain 4 - Yeast codon optimized DNA sequence without bacterial signal

ttccactacgacagaaacaacattgctgtcggtgctgacgaatccgttgtcaaggaagccc
acagagaagtcatcaactcctccaccgaaggtttgttgttgaacatcgacaaggacatcag
aaagatcttgtccggttacatcgtcgaaattgaagacaccgaaggtttgaaggaagtcatc
aacgacagatacgacatgttgaacatttcctctttgagacaagacggtaagaccttcatcg
acttcaagaagtacaacgacaagttgccattgtacatttccaacccaaactacaaggtcaa
cgtttacgctgtcactaaggaaaacaccatcatcaacccatccgaaaacggtgacacttcc
accaacggtatcaagaagattttgatcttctctaagaagggttacgaaattggt

Domain 4- Translated protein without signal sequence

FHYDRNNIAVGADESVVKEAHREVINSSTEGLLLNIDKDIRKILSGYIVEIED
TEGLKEVINDRYDMLNISSLRQDGKTFIDFKKYNDKLPLYISNPNYKVNVYAV
TKENTIINPSENGDTSTNGIKKILIFSKKGYEIG

FIGURE 9

DNA sequence and amino acid translation product of yeast codon-optimized B. anthracis PA83 gene

DNA sequence

```
    1   GAAGTTAAGC AAGAAAACAG ATTGTTGAAC GAATCTGAAT CCTCTTCCCA AGGTTTGTTG
        CTTCAATTCG TTCTTTTGTC TAACAACTTG CTTAGACTTA GGAGAAGGGT TCCAAACAAC
   61   GGTTACTACT TCTCCGACTT GAACTTCCAA GCTCCAATGG TCGTCACCTC CTCTACTACC
        CCAATGATGA AGAGGCTGAA CTTGAAGGTT CGAGGTTACC AGCAGTGGAG GAGATGATGG
  121   GGTGACTTGT CCATCCCATC CTCCGAATTA GAGAACATCC CATCCGAAAA CCAATACTTC
        CCACTGAACA GGTAGGGTAG GAGGCTTAAT CTCTTGTAGG GTAGGCTTTT GGTTATGAAG
  181   CAATCCGCTA TCTGGTCCGG CTTCATCAAG GTCAAGAAGT CCGACGAATA CACTTTCGCT
        GTTAGGCGAT AGACCAGGCC GAAGTAGTTC CAGTTCTTCA GGCTGCTTAT GTGAAAGCGA
  241   ACCTCCGCTG ACAACCACGT TACTATGTGG GTCGACGACC AAGAAGTCAT CAACAAGGCT
        TGGAGGCGAC TGTTGGTGCA ATGATACACC CAGCTGCTGG TTCTTCAGTA GTTGTTCCGA
  301   TCCAACTCCA ACAAGATCAG ATTGGAAAAG GGTAGATTGT ACCAAATTAA GATCCAATAC
        AGGTTGAGGT TGTTCTAGTC TAACCTTTTC CCATCTAACA TGGTTTAATT CTAGGTTATG
  361   CAAAGAGAAA ACCCAACCGA AAAGGGTTTG GACTTCAAGT TGTACTGGAC CGACTCCCAA
        GTTTCTCTTT TGGGTTGGCT TTTCCCAAAC CTGAAGTTCA ACATGACCTG GCTGAGGGTT
  421   AACAAGAAGG AAGTCATCTC CTCTGACAAC TTGCAATTGC CAGAATTGAA GCAAAAGTCC
        TTGTTCTTCC TTCAGTAGAG GAGACTGTTG AACGTTAACG GTCTTAACTT CGTTTTCAGG
  481   TCCAACTCCA GAAAGAAGAG ATCCACCTCC GCTGGTCCAA CTGTTCCAGA CAGAGACAAC
        AGGTTGAGGT CTTTCTTCTT CAGGTGGAGG CGACCAGGTT GACAAGGTCT GTCTCTGTTG
  541   GACGGTATTC CAGACTCCTT GGAAGTCGAA GGTTACACCG TCGACGTCAA GAACAAGAGA
        CTGCCATAAG GTCTGAGGAA CCTTCAGCTT CCAATGTGGC AGCTGCAGTT CTTGTTCTCT
  601   ACCTTCTTGT CTCCATGGAT CTCCAACATT CACGAAAAGA AGGGTTTGAC CAAGTACAAG
        TGGAAGAACA GAGGTACCTA GAGGTTGTAA GTGCTTTTCT TCCCAAACTG GTTCATGTTC
  661   TCTTCCCCAG AAAAGTGGTC TACTGCTTCC GACCCATACT CCGACTTCGA AAAGGTCACC
        AGAAGGGGTC TTTTCACCAG ATGACGAAGG CTGGGTATGA GGCTGAAGCT TTTCCAGTGG
  721   GGTAGAATCG ACAAGAACGT CTCCCCAGAA GCTAGACACC CATTGGTTGC TGCTTACCCA
        CCATCTTAGC TGTTCTTGCA GAGGGGTCTT CGATCTGTGG GTAACCAACG ACGAATGGGT
  781   ATTGTCCACG TTGACATGGA AAACATCATT TTGTCCAAGA ACGAAGACCA ATCCACCCAA
        TAACAGGTGC AACTGTACCT TTTGTAGTAA AACAGGTTCT TGCTTCTGGT TAGGTGGGTT
  841   AACACGGACT CCGAAACCAG AACTATCTCT AAGAACACCT CCACTTCTAG AACCCACACT
        TTGTGCCTGA GGCTTTGGTC TTGATAGAGA TTCTTGTGGA GGTGAAGATC TTGGGTGTGA
  901   TCCGAAGTCC ACGGTAACGC TGAAGTTCAC GCTTCTTTCT TCGACATTGG TGGTTCCGTC
        AGGCTTCAGG TGCCATTGCG ACTTCAAGTG CGAAGAAAGA AGCTGTAACC ACCAAGGCAG
  961   TCTGCTGGTT TCTCCAACTC CAACTCCTCC ACCGTCGCTA TCGACCACTC TTTGTCCTTG
        AGACGACCAA AGAGGTTGAG GTTGAGGAGG TGGCAGCGAT AGCTGGTGAG AAACAGGAAC
 1021   GCTGGTGAAA GAACTTGGGC TGAAACCATG GGTTTGAACA CTGCTGACAC CGCTAGATTG
        CGACCACTTT CTTGAACCCG ACTTTGGTAC CCAAACTTGT GACGACTGTG GCGATCTAAC
 1081   AACGCTAACA TTAGATACGT CAACACTGGT ACCGCTCCAA TCTACAACGT CTTGCCAACC
        TTGCGATTGT AATCTATGCA GTTGTGACCA TGGCGAGGTT AGATGTTGCA GAACGGTTGG
 1141   ACCTCCTTGG TCTTGGGTAA GAACCAAACC TTGGCTACTA TCAAGGCTAA GGAAAACCAA
        TGGAGGAACC AGAACCCATT CTTGGTTTGG AACCGATGAT AGTTCCGATT CCTTTTGGTT
 1201   TTGTCCCAAA TCTTGGCTCC AAACAACTAC TACCCATCCA AGAACTTGGC TCCAATCGCT
        AACAGGGTTT AGAACCGAGG TTTGTTGATG ATGGGTAGGT TCTTGAACCG AGGTTAGCGA
 1261   TTGAACGCTC AAGACGACTT CTCCTCTACT CCAATCACCA TGAACTACAA CCAATTCTTG
        AACTTGCGAG TTCTGCTGAA GAGGAGATGA GGTTAGTGGT ACTTGATGTT GGTTAAGAAC
 1321   GAATTGGAAA AGACTAAGCA ATTGCGTTTG GACACCGACC AAGTTTACGG TAACATTGCT
        CTTAACCTTT TCTGATTCGT TAACGCAAAC CTGTGGCTGG TTCAAATGCC ATTGTAACGA
 1381   ACTTACAACT TCGAAAACGG TAGAGTCAGA GTCGACACCG GTTCCAACTG GTCTGAAGTC
        TGAATGTTGA AGCTTTTGCC ATCTCAGTCT CAGCTGTGGC CAAGGTTGAC CAGACTTCAG
 1441   TTGCCACAAA TCCAAGAAAC CACCGCTAGA ATCATCTTCA ACGGTAAGGA CTTGAACTTG
        AACGGTGTTT AGGTTCTTTG GTGGCGATCT TAGTAGAAGT TGCCATTCCT GAACTTGAAC
 1501   GTTGAAAGAA GAATTGCTGC TGTCAACCCA TCCGACCCAT TGGAAACCAC CAAGCCAGAC
        CAACTTTCTT CTTAACGACG ACAGTTGGGT AGGCTGGGTA ACCTTTGGTG GTTCGGTCTG
 1561   ATGACCTTGA AGGAAGCTTT GAAGATCGCT TTCGGTTTCA ACGAACCAAA CGGTAACTTG
        TACTGGAACT TCCTTCGAAA CTTCTAGCGA AAGCCAAAGT TGCTTGGTTT GCCATTGAAC
```

FIGURE 10A – 1

```
1621    CAATACCAAG GTAAGGACAT CACCGAATTC GACTTCAACT TCGACCAACA AACCTCTCAA
        GTTATGGTTC CATTCCTGTA GTGGCTTAAG CTGAAGTTGA AGCTGGTTGT TTGGAGAGTT
1681    AACATCAAGA ACCAATTGGC TGAATTGAAC GCTACCAACA TTTACACTGT TTTGGACAAG
        TTGTAGTTCT TGGTTAACCG ACTTAACTTG CGATGGTTGT AAATGTGACA AAACCTGTTC
1741    ATCAAGTTGA ACGCCAAGAT GAACATCTTG ATCAGAGACA AGAGATTCCA CTACGACAGA
        TAGTTCAACT TGCGGTTCTA CTTGTAGAAC TAGTCTCTGT TCTCTAAGGT GATGCTGTCT
1801    AACAACATTG CTGTCGGTGC TGACGAATCC GTTGTCAAGG AAGCCCACAG AGAAGTCATC
        TTGTTGTAAC GACAGCCACG ACTGCTTAGG CAACAGTTCC TTCGGGTGTC TCTTCAGTAG
1861    AACTCCTCCA CCGAAGGTTT GTTGTTGAAC ATCGACAAGG ACATCAGAAA GATCTTGTCC
        TTGAGGAGGT GGCTTCCAAA CAACAACTTG TAGCTGTTCC TGTAGTCTTT CTAGAACAGG
1921    GGTTACATCG TCGAAATTGA AGACACCGAA GGTTTGAAGG AAGTCATCAA CGACAGATAC
        CCAATGTAGC AGCTTTAACT TCTGTGGCTT CCAAACTTCC TTCAGTAGTT GCTGTCTATG
1981    GACATGTTGA ACATTTCCTC TTTGAGACAA GACGGTAAGA CCTTCATCGA CTTCAAGAAG
        CTGTACAACT TGTAAAGGAG AAACTCTGTT CTGCCATTCT GGAAGTAGCT GAAGTTCTTC
2041    TACAACGACA AGTTGCCATT GTACATTTCC AACCCAAACT ACAAGGTCAA CGTTTACGCT
        ATGTTGCTGT TCAACGGTAA CATGTAAAGG TTGGGTTTGA TGTTCCAGTT GCAAATGCGA
2101    GTCACTAAGG AAAACACCAT CATCAACCCA TCCGAAAACG GTGACACTTC CACCAACGGT
        CAGTGATTCC TTTTGTGGTA GTAGTTGGGT AGGCTTTTGC CACTGTGAAG GTGGTTGCCA
2161    ATCAAGAAGA TTTTGATCTT CTCTAAGAAG GGTTACGAAA TTGGT
```

FIGURE 10A – 2

Translation of Yeast codon optimized DNA sequence without leader

```
  1  EVKQENRLLN ESESSSQGLL GYYFSDLNFQ APMVVTSSTT GDLSIPSSEL
 51  ENIPSENQYF QSAIWSGFIK VKKSDEYTFA TSADNHVTMW VDDQEVINKA
101  SNSNKIRLEK GRLYQIKIQY QRENPTEKGL DFKLYWTDSQ NKKEVISSDN
151  LQLPELKQKS SNSRKKRSTS AGPTVPDRDN DGIPDSLEVE GYTVDVKNKR
201  TFLSPWISNI HEKKGLTKYK SSPEKWSTAS DPYSDFEKVT GRIDKNVSPE
251  ARHPLVAAYP IVHVDMENII LSKNEDQSTQ NTDSETRTIS KNTSTSRTHT
301  SEVHGNAEVH ASFFDIGGSV SAGFSNSNSS TVAIDHSLSL AGERTWAETM
351  GLNTADTARL NANIRYVNTG TAPIYNVLPT TSLVLGKNQT LATIKAKENQ
401  LSQILAPNNY YPSKNLAPIA LNAQDDFSST PITMNYNQFL ELEKTKQLRL
451  DTDQVYGNIA TYNFENGRVR VDTGSNWSEV LPQIQETTAR IIFNGKDLNL
501  VERRIAAVNP SDPLETTKPD MTLKEALKIA FGFNEPNGNL QYQGKDITEF
551  DFNFDQQTSQ NIKNQLAELN ATNIYTVLDK IKLNAKMNIL IRDKRFHYDR
601  NNIAVGADES VVKEAHREVI NSSTEGLLLN IDKDIRKILS GYIVEIEDTE
651  GLKEVINDRY DMLNISSLRQ DGKTFIDFKK YNDKLPLYIS NPNYKVNVYA
701  VTKENTIINP SENGDTSTNG IKKILIFSKK GYEIG
```

FIGURE 10B

DNA sequence and amino acid translation product of yeast codon-optimized B. anthracis PA83 gene with modified furin cleavage site

DNA sequence

```
    1   GAAGTTAAGC AAGAAAACAG ATTGTTGAAC GAATCTGAAT CCTCTTCCCA AGGTTTGTTG
        CTTCAATTCG TTCTTTTGTC TAACAACTTG CTTAGACTTA GGAGAAGGGT TCCAAACAAC
   61   GGTTACTACT TCTCCGACTT GAACTTCCAA GCTCCAATGG TCGTCACCTC CTCTACTACC
        CCAATGATGA AGAGGCTGAA CTTGAAGGTT CGAGGTTACC AGCAGTGGAG GAGATGATGG
  121   GGTGACTTGT CCATCCCATC CTCCGAATTA GAGAACATCC CATCCGAAAA CCAATACTTC
        CCACTGAACA GGTAGGGTAG GAGGCTTAAT CTCTTGTAGG GTAGGCTTTT GGTTATGAAG
  181   CAATCCGCTA TCTGGTCCGG CTTCATCAAG GTCAAGAAGT CCGACGAATA CACTTTCGCT
        GTTAGGCGAT AGACCAGGCC GAAGTAGTTC CAGTTCTTCA GGCTGCTTAT GTGAAAGCGA
  241   ACCTCCGCTG ACAACCACGT TACTATGTGG GTCGACGACC AAGAAGTCAT CAACAAGGCT
        TGGAGGCGAC TGTTGGTGCA ATGATACACC CAGCTGCTGG TTCTTCAGTA GTTGTTCCGA
  301   TCCAACTCCA ACAAGATCAG ATTGGAAAAG GGTAGATTGT ACCAAATTAA GATCCAATAC
        AGGTTGAGGT TGTTCTAGTC TAACCTTTTC CCATCTAACA TGGTTTAATT CTAGGTTATG
  361   CAAAGAGAAA ACCCAACCGA AAAGGGTTTG GACTTCAAGT TGTACTGGAC CGACTCCCAA
        GTTTCTCTTT TGGGTTGGCT TTTCCCAAAC CTGAAGTTCA ACATGACCTG GCTGAGGGTT
  421   AACAAGAAGG AAGTCATCTC CTCTGACAAC TTGCAATTGC CAGAATTGAA GCAAAAGTCC
        TTGTTCTTCC TTCAGTAGAG GAGACTGTTG AACGTTAACG GTCTTAACTT CGTTTTCAGG
  481   TCCAACTCCA GAAAGAAGAA GTCCACCTCC GCTGGTCCAA CTGTTCCAGA CAGAGACAAC
        AGGTTGAGGT CTTTCTTCTT CAGGTGGAGG CGACCAGGTT GACAAGGTCT GTCTCTGTTG
  541   GACGGTATTC CAGACTCCTT GGAAGTCGAA GGTTACACCG TCGACGTCAA GAACAAGAGA
        CTGCCATAAG GTCTGAGGAA CCTTCAGCTT CCAATGTGGC AGCTGCAGTT CTTGTTCTCT
  601   ACCTTCTTGT CTCCATGGAT CTCCAACATT CACGAAAAGA AGGGTTTGAC CAAGTACAAG
        TGGAAGAACA GAGGTACCTA GAGGTTGTAA GTGCTTTTCT TCCCAAACTG GTTCATGTTC
  661   TCTTCCCCAG AAAAGTGGTC TACTGCTTCC GACCCATACT CCGACTTCGA AAAGGTCACC
        AGAAGGGGTC TTTTCACCAG ATGACGAAGG CTGGGTATGA GGCTGAAGCT TTTCCAGTGG
  721   GGTAGAATCG ACAAGAACGT CTCCCCAGAA GCTAGACACC CATTGGTTGC TGCTTACCCA
        CCATCTTAGC TGTTCTTGCA GAGGGGTCTT CGATCTGTGG GTAACCAACG ACGAATGGGT
  781   ATTGTCCACG TTGACATGGA AAACATCATT TTGTCCAAGA ACGAAGACCA ATCCACCCAA
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  841   AACACGGACT CCGAAACCAG AACTATCTCT AAGAACACCT CCACTTCTAG AACCCACACT
        TTGTGCCTGA GGCTTTGGTC TTGATAGAGA TTCTTGTGGA GGTGAAGATC TTGGGTGTGA
  901   TCCGAAGTCC ACGGTAACGC TGAAGTTCAC GCTTCTTTCT TCGACATTGG TGGTTCCGTC
        AGGCTTCAGG TGCCATTGCG ACTTCAAGTG CGAAGAAAGA AGCTGTAACC ACCAAGGCAG
  961   TCTGCTGGTT TCTCCAACTC CAACTCCTCC ACCGTCGCTA TCGACCACTC TTTGTCCTTG
        AGACGACCAA AGAGGTTGAG GTTGAGGAGG TGGCAGCGAT AGCTGGTGAG AAACAGGAAC
 1021   GCTGGTGAAA GAACTTGGGC TGAAACCATG GGTTTGAACA CTGCTGACAC CGCTAGATTG
        CGACCACTTT CTTGAACCCG ACTTTGGTAC CCAAACTTGT GACGACTGTG GCGATCTAAC
 1081   AACGCTAACA TTAGATACGT CAACACTGGT ACCGCTCCAA TCTACAACGT CTTGCCAACC
        TTGCGATTGT AATCTATGCA GTTGTGACCA TGGCGAGGTT AGATGTTGCA GAACGGTTGG
 1141   ACCTCCTTGG TCTTGGGTAA GAACCAAACC TTGGCTACTA TCAAGGCTAA GGAAAACCAA
        TGGAGGAACC AGAACCCATT CTTGGTTTGG AACCGATGAT AGTTCCGATT CCTTTTGGTT
 1201   TTGTCCCAAA TCTTGGCTCC AAACAACTAC TACCCATCCA AGAACTTGGC TCCAATCGCT
        AACAGGGTTT AGAACCGAGG TTTGTTGATG ATGGGTAGGT TCTTGAACCG AGGTTAGCGA
 1261   TTGAACGCTC AAGACGACTT CTCCTCTACT CCAATCACCA TGAACTACAA CCAATTCTTG
        AACTTGCGAG TTCTGCTGAA GAGGAGATGA GGTTAGTGGT ACTTGATGTT GGTTAAGAAC
 1321   GAATTGGAAA AGACTAAGCA ATTGCGTTTG GACACCGACC AAGTTTACGG TAACATTGCT
        CTTAACCTTT TCTGATTCGT TAACGCAAAC CTGTGGCTGG TTCAAATGCC ATTGTAACGA
 1381   ACTTACAACT TCGAAAACGG TAGAGTCAGA GTCGACACCG GTTCCAACTG GTCTGAAGTC
        TGAATGTTGA AGCTTTTGCC ATCTCAGTCT CAGCTGTGGC CAAGGTTGAC CAGACTTCAG
 1441   TTGCCACAAA TCCAAGAAAC CACCGCTAGA ATCATCTTCA ACGGTAAGGA CTTGAACTTG
        AACGGTGTTT AGGTTCTTTG GTGGCGATCT TAGTAGAAGT TGCCATTCCT GAACTTGAAC
 1501   GTTGAAAGAA GAATTGCTGC TGTCAACCCA TCCGACCCAT TGGAAACCAC CAAGCCAGAC
        CAACTTTCTT CTTAACGACG ACAGTTGGGT AGGCTGGGTA ACCTTTGGTG GTTCGGTCTG
```

FIGURE 11A – 1

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1561    ATGACCTTGA AGGAAGCTTT GAAGATCGCT TTCGGTTTCA ACGAACCAAA CGGTAACTTG
        TACTGGAACT TCCTTCGAAA CTTCTAGCGA AAGCCAAAGT TGCTTGGTTT GCCATTGAAC
 1621   CAATACCAAG GTAAGGACAT CACCGAATTC GACTTCAACT TCGACCAACA AACCTCTCAA
        GTTATGGTTC CATTCCTGTA GTGGCTTAAG CTGAAGTTGA AGCTGGTTGT TTGGAGAGTT
 1681   AACATCAAGA ACCAATTGGC TGAATTGAAC GCTACCAACA TTTACACTGT TTTGGACAAG
        TTGTAGTTCT TGGTTAACCG ACTTAACTTG CGATGGTTGT AAATGTGACA AAACCTGTTC
 1741   ATCAAGTTGA ACGCCAAGAT GAACATCTTG ATCAGAGACA AGAGATTCCA CTACGACAGA
        TAGTTCAACT TGCGGTTCTA CTTGTAGAAC TAGTCTCTGT TCTCTAAGGT GATGCTGTCT
 1801   AACAACATTG CTGTCGGTGC TGACGAATCC GTTGTCAAGG AAGCCCACAG AGAAGTCATC
        TTGTTGTAAC GACAGCCACG ACTGCTTAGG CAACAGTTCC TTCGGGTGTC TCTTCAGTAG
 1861   AACTCCTCCA CCGAAGGTTT GTTGTTGAAC ATCGACAAGG ACATCAGAAA GATCTTGTCC
        TTGAGGAGGT GGCTTCCAAA CAACAACTTG TAGCTGTTCC TGTAGTCTTT CTAGAACAGG
 1921   GGTTACATCG TCGAAATTGA AGACACCGAA GGTTTGAAGG AAGTCATCAA CGACAGATAC
        CCAATGTAGC AGCTTTAACT TCTGTGGCTT CCAAACTTCC TTCAGTAGTT GCTGTCTATG
 1981   GACATGTTGA ACATTTCCTC TTTGAGACAA GACGGTAAGA CCTTCATCGA CTTCAAGAAG
        CTGTACAACT TGTAAAGGAG AAACTCTGTT CTGCCATTCT GGAAGTAGCT GAAGTTCTTC
 2041   TACAACGACA AGTTGCCATT GTACATTTCC AACCCAAACT ACAAGGTCAA CGTTTACGCT
        ATGTTGCTGT TCAACGGTAA CATGTAAAGG TTGGGTTTGA TGTTCCAGTT GCAAATGCGA
 2101   GTCACTAAGG AAAACACCAT CATCAACCCA TCCGAAAACG GTGACACTTC CACCAACGGT
        CAGTGATTCC TTTTGTGGTA GTAGTTGGGT AGGCTTTTGC CACTGTGAAG GTGGTTGCCA
 2161   ATCAAGAAGA TTTTGATCTT CTCTAAGAAG GGTTACGAAA TTGGT
```

FIGURE 11A – 2

Translated

```
  1  EVKQENRLLN ESESSSQGLL GYYFSDLNFQ APMVVTSSTT GDLSIPSSEL
 51  ENIPSENQYF QSAIWSGFIK VKKSDEYTFA TSADNHVTMW VDDQEVINKA
101  SNSNKIRLEK GRLYQIKIQY QRENPTEKGL DFKLYWTDSQ NKKEVISSDN
151  LQLPELKQKS SNSRKKKSTS AGPTVPDRDN DGIPDSLEVE GYTVDVKNKR
201  TFLSPWISNI HEKKGLTKYK SSPEKWSTAS DPYSDFEKVT GRIDKNVSPE
251  ARHPLVAAYP IVHVDMENII LSKNEDQSTQ NTDSETRTIS KNTSTSRTHT
301  SEVHGNAEVH ASFFDIGGSV SAGFSNSNSS TVAIDHSLSL AGERTWAETM
351  GLNTADTARL NANIRYVNTG TAPIYNVLPT TSLVLGKNQT LATIKAKENQ
401  LSQILAPNNY YPSKNLAPIA LNAQDDFSST PITMNYNQFL ELEKTKQLRL
451  DTDQVYGNIA TYNFENGRVR VDTGSNWSEV LPQIQETTAR IIFNGKDLNL
501  VERRIAAVNP SDPLETTKPD MTLKEALKIA FGFNEPNGNL QYQGKDITEF
551  DFNFDQQTSQ NIKNQLAELN ATNIYTVLDK IKLNAKMNIL IRDKRFHYDR
601  NNIAVGADES VVKEAHREVI NSSTEGLLLN IDKDIRKILS GYIVEIEDTE
651  GLKEVINDRY DMLNISSLRQ DGKTFIDFKK YNDKLPLYIS NPNYKVNVYA
701  VTKENTIINP SENGDTSTNG IKKILIFSKK GYEIG
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FIGURE 11B

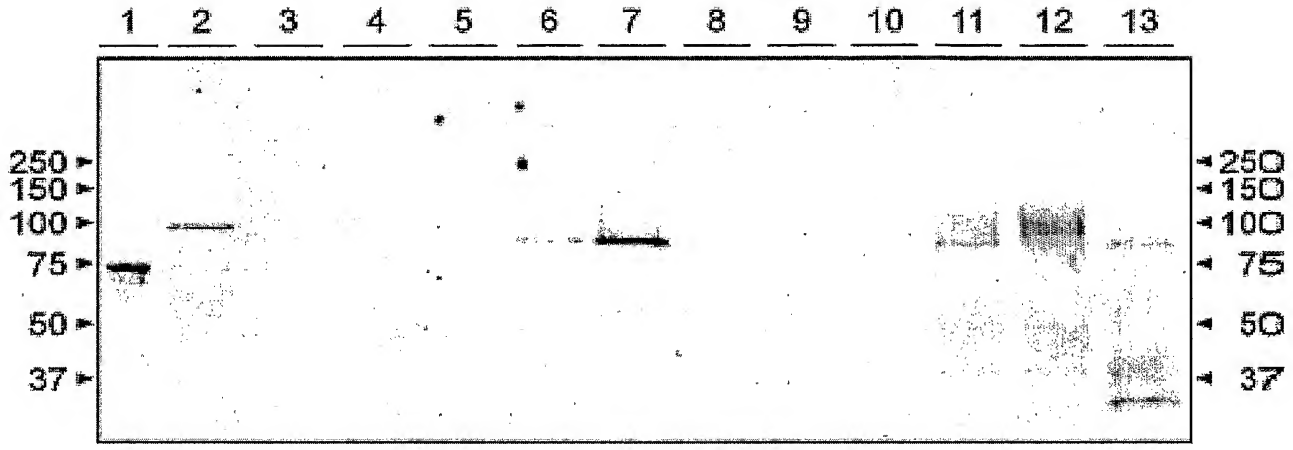

FIGURE 12

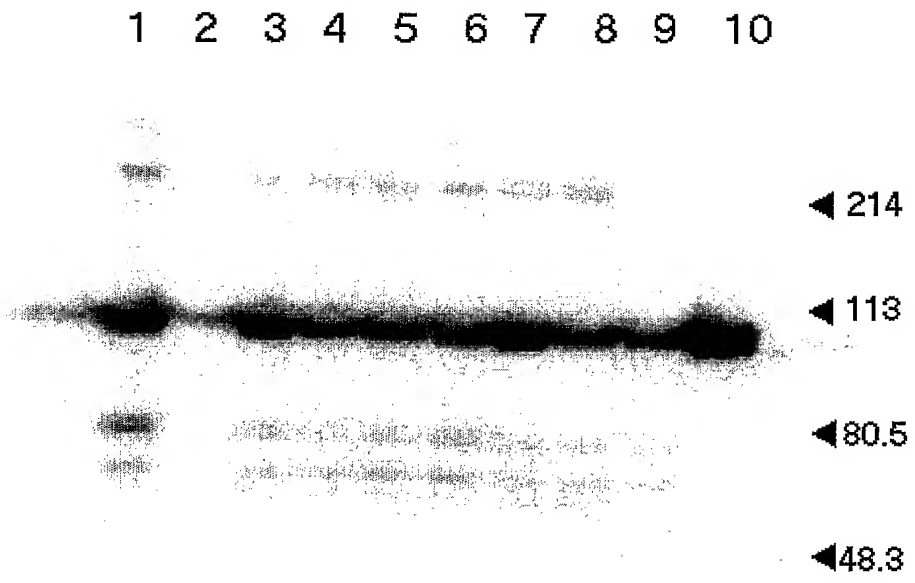

FIGURE 13

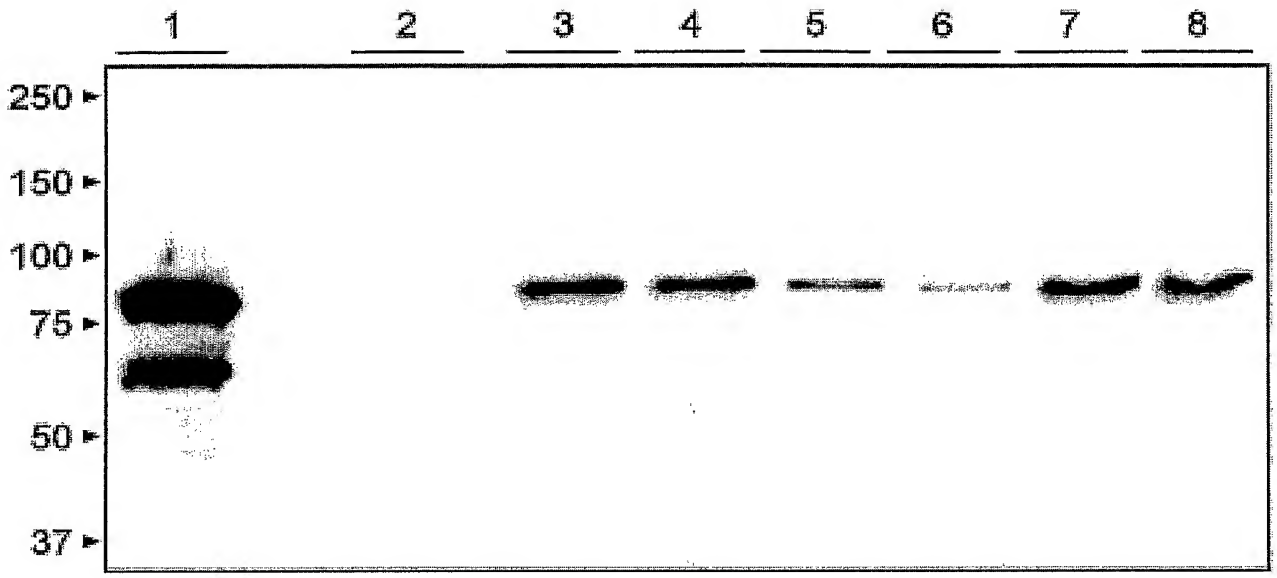

FIGURE 14

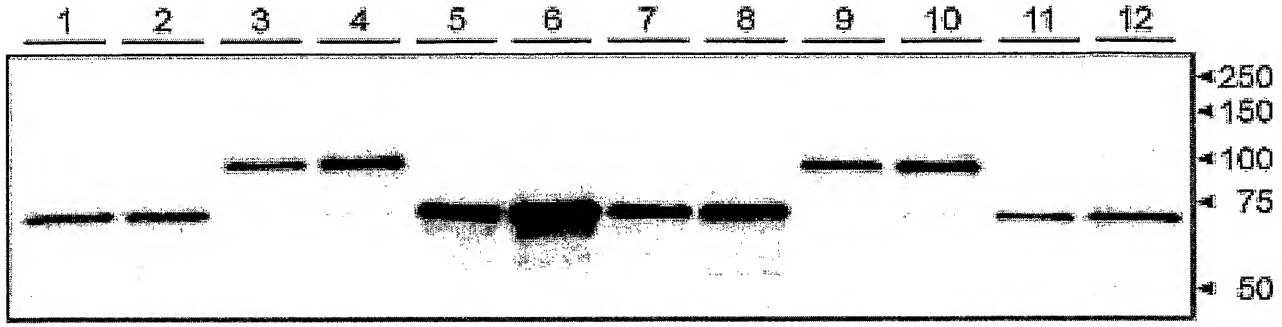

FIGURE 15

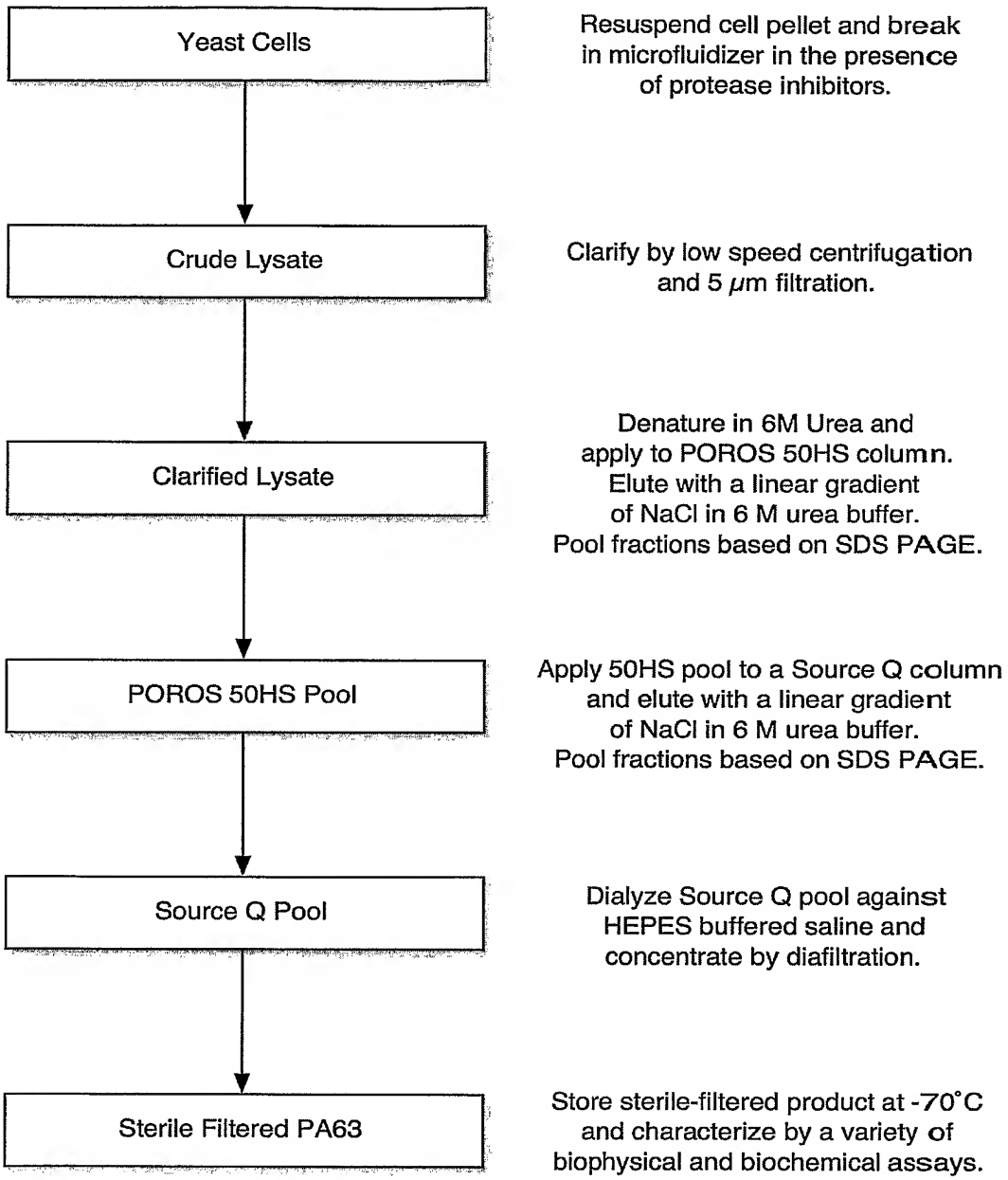
Yeast Cells
Resuspend cell pellet and break in microfluidizer in the presence of protease inhibitors.
Crude Lysate
Clarify by low speed centrifugation and 5 μm filtration.
Clarified Lysate
Denature in 6M Urea and apply to POROS 50HS column. Elute with a linear gradient of NaCl in 6 M urea buffer. Pool fractions based on SDS PAGE.
POROS 50HS Pool
Apply 50HS pool to a Source Q column and elute with a linear gradient of NaCl in 6 M urea buffer. Pool fractions based on SDS PAGE.
Source Q Pool
Dialyze Source Q pool against HEPES buffered saline and concentrate by diafiltration.
Sterile Filtered PA63
Store sterile-filtered product at -70°C and characterize by a variety of biophysical and biochemical assays.

FIGURE 16

A. SDS PAGE Analysis of 50HS Chromatography

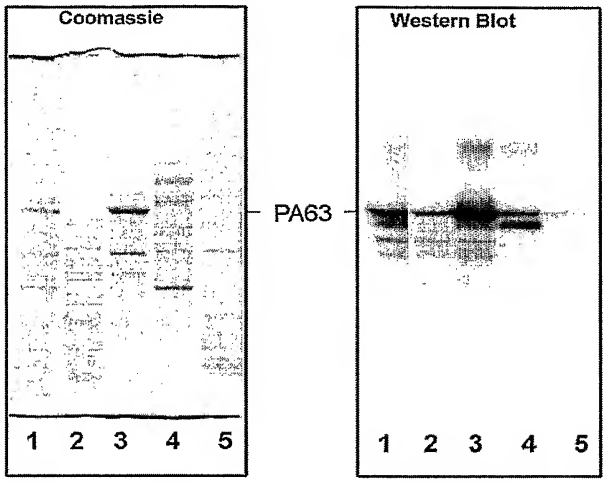

FIGURE 17A

B. SDS PAGE Analysis of Source Q Chromatography

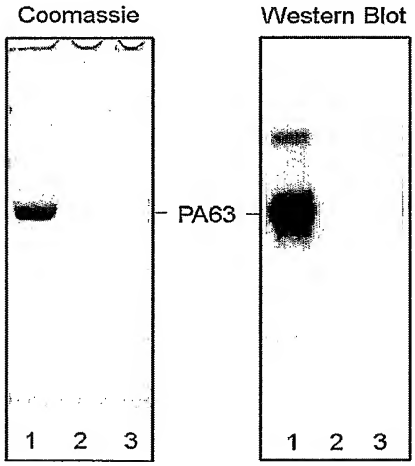

FIGURE 17B

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acaataagta aaaatacttc tacaagtagg acacatacta gtgaagtaca tggaaatgca 420

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gaaacaatgg gtttaaatac cgctgataca gcaagattaa atgccaatat tagatatgta 600
aatactggga cggctccaat ctacaacgtg ttaccaacga cttcgttagt gttaggaaaa 660
aatcaaacac tcgcgacaat taaagctaag gaaaaccaat taagtcaaat acttgcacct 720
aataattatt atccttctaa aaacttggcg ccaatcgcat taaatgcaca agacgatttc 780
agttctactc caattacaat gaattacaat caatttcttg agttagaaaa aacgaaacaa 840
ttaagattag atacggatca agtatatggg aatatagcaa catacaattt tgaaaatgga 900
agagtgaggg tggatacagg ctcgaactgg agtgaagtgt taccgcaaat tcaagaaaca 960
actgcacgta tcatttttaa tggaaaagat ttaaatctgg tagaaaggcg gatagcggcg 1020
gttaatccta gtgatccatt agaaacgact aaaccggata tgacattaaa agaagccctt 1080
aaaatagcat ttggatttaa cgaaccgaat ggaaacttac aatatcaagg gaaagacata 1140
accgaatttg attttaattt cgatcaacaa acatctcaaa atatcaagaa tcagttagcg 1200
gaattaaacg caactaacat atatactgta ttagataaaa tcaaattaaa tgcaaaaatg 1260
aatattttaa taagagataa acgttttcat tatgatagaa ataacatagc agttggggcg 1320
gatgagtcag tagttaagga ggctcataga gaagtaatta attcgtcaac agagggatta 1380
ttgttaaata ttgataagga tataagaaaa atattatcag gttatattgt agaaattgaa 1440
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ttacggcaag atggaaaaac atttatagat tttaaaaaat ataatgataa attaccgtta 1560
tatataagta atcccaatta taaggtaaat gtatatgctg ttactaaaga aaacactatt 1620
attaatccta gtgagaatgg ggatactagt accaacggga tcaagaaaat tttaatcttt 1680
tctaaaaaag gctatgagat agga                                        1704
```

<210> 48
<211> 571
<212> PRT
<213> Bacillus anthracis

<400> 48

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Ser Thr Ser Ala Gly Pro Thr Val Pro Asp Arg Asp Asn Asp Gly Ile
 1               5                  10                  15
Pro Asp Ser Leu Glu Val Glu Gly Tyr Thr Val Asp Val Lys Asn Lys
            20                  25                  30
Arg Thr Phe Leu Ser Pro Trp Ile Ser Asn Ile His Glu Lys Lys Gly
        35                  40                  45
Leu Thr Lys Tyr Lys Ser Ser Pro Glu Lys Trp Ser Thr Ala Ser Asp
    50                  55                  60
Pro Tyr Ser Asp Phe Glu Lys Val Thr Gly Arg Ile Asp Lys Asn Val
65                  70                  75                  80
Ser Pro Glu Ala Arg His Pro Leu Val Ala Ala Tyr Pro Ile Val His
                85                  90                  95
Val Asp Met Glu Asn Ile Ile Leu Ser Lys Asn Glu Asp Gln Ser Thr
            100                 105                 110
Gln Asn Thr Asp Ser Glu Thr Arg Thr Ile Ser Lys Asn Thr Ser Thr
        115                 120                 125
Ser Arg Thr His Thr Ser Glu Val His Gly Asn Ala Glu Val His Ala
    130                 135                 140
Ser Phe Phe Asp Ile Gly Gly Ser Val Ser Ala Gly Phe Ser Asn Phe
145                 150                 155                 160
Ile Gly Ser Asn Ser Ser Thr Val Ala Ile Asp His Ser Leu Ser Leu
                165                 170                 175
Ala Gly Glu Arg Thr Trp Ala Glu Thr Met Gly Leu Asn Thr Ala Asp
            180                 185                 190
Thr Ala Arg Leu Asn Ala Asn Ile Arg Tyr Val Asn Thr Gly Thr Ala
        195                 200                 205
```

```
Pro Ile Tyr Asn Val Leu Pro Thr Thr Ser Leu Val Leu Gly Lys Asn
    210                 215                 220
Gln Thr Leu Ala Thr Ile Lys Ala Lys Glu Asn Gln Leu Ser Gln Ile
225                 230                 235                 240
Leu Ala Pro Asn Asn Tyr Tyr Pro Ser Lys Asn Leu Ala Pro Ile Ala
                245                 250                 255
Leu Asn Ala Gln Asp Asp Phe Ser Ser Thr Pro Ile Thr Met Asn Tyr
            260                 265                 270
Asn Gln Phe Leu Glu Leu Glu Lys Thr Lys Gln Leu Arg Leu Asp Thr
        275                 280                 285
Asp Gln Val Tyr Gly Asn Ile Ala Thr Tyr Asn Phe Glu Asn Gly Arg
    290                 295                 300
Val Arg Val Asp Thr Gly Ser Asn Trp Ser Glu Val Leu Pro Gln Ile
305                 310                 315                 320
Gln Glu Thr Thr Ala Arg Ile Ile Phe Asn Gly Lys Asp Leu Asn Leu
                325                 330                 335
Val Glu Arg Arg Ile Ala Ala Val Asn Pro Ser Asp Pro Leu Glu Thr
            340                 345                 350
Thr Lys Pro Asp Met Thr Leu Lys Glu Ala Leu Lys Ile Ala Phe Gly
        355                 360                 365
Phe Asn Glu Pro Asn Gly Asn Leu Gln Tyr Gln Gly Lys Asp Ile Thr
    370                 375                 380
Glu Phe Asp Phe Asn Phe Asp Gln Gln Thr Ser Gln Asn Ile Lys Asn
385                 390                 395                 400
Gln Leu Ala Glu Leu Asn Ala Thr Asn Ile Tyr Thr Val Leu Asp Lys
                405                 410                 415
Ile Lys Leu Asn Ala Lys Met Asn Ile Leu Ile Arg Asp Lys Arg Phe
            420                 425                 430
His Tyr Asp Arg Asn Asn Ile Ala Val Gly Ala Asp Glu Ser Val Val
        435                 440                 445
Lys Glu Ala His Arg Glu Val Ile Asn Ser Ser Thr Glu Gly Leu Leu
    450                 455                 460
Leu Asn Ile Asp Lys Asp Ile Arg Lys Ile Leu Ser Gly Tyr Ile Val
465                 470                 475                 480
Glu Ile Glu Asp Thr Glu Gly Leu Lys Glu Val Ile Asn Asp Arg Tyr
                485                 490                 495
Asp Met Leu Asn Ile Ser Ser Leu Arg Gln Asp Gly Lys Thr Phe Ile
            500                 505                 510
Asp Phe Lys Lys Tyr Asn Asp Lys Leu Pro Leu Tyr Ile Ser Asn Pro
        515                 520                 525
Asn Tyr Lys Val Asn Val Tyr Ala Val Thr Lys Glu Asn Thr Ile Ile
    530                 535                 540
Asn Pro Ser Glu Asn Gly Asp Thr Ser Thr Asn Gly Ile Lys Lys Ile
545                 550                 555                 560
Leu Ile Phe Ser Lys Lys Gly Tyr Glu Ile Gly
                565                 570
```

<210> 49
<211> 1704
<212> DNA
<213> Bacillus anthracis

<400> 49
tccacctccg ctggtccaac tgttccagac agagacaacg acggtattcc agactccttg 60
gaagtcgaag gttacaccgt cgacgtcaag aacaagagaa ccttcttgtc tccatggatc 120

```
tccaacattc acgaaaagaa gggtttgacc aagtacaagt cttccccaga aaagtggtct 180
actgcttccg acccatactc cgacttcgaa aaggtcaccg gtagaatcga caagaacgtc 240
tccccagaag ctagacaccc attggttgct gcttacccaa ttgtccacgt tgacatggaa 300
aacatcattt tgtccaagaa cgaagaccaa tccacccaaa acacggactc cgaaaccaga 360
actatctcta agaacacctc cacttctaga acccacactt ccgaagtcca cggtaacgct 420
gaagttcacg cttctttctt cgacattggt ggttccgtct ctgctggttt ctccaactcc 480
aactcctcca ccgtcgctat cgaccactct ttgtccttgg ctggtgaaag aacttgggct 540
gaaaccatgg gtttgaacac tgctgacacc gctagattga acgctaacat tagatacgtc 600
aacactggta ccgctccaat ctacaacgtc ttgccaacca cctccttggt cttgggtaag 660
aaccaaacct tggctactat caaggctaag gaaaaccaat tgtcccaaat cttggctcca 720
aacaactact acccatccaa gaacttggct ccaatcgctt tgaacgctca agacgacttc 780
tcctctactc caatcaccat gaactacaac caattcttgg aattggaaaa gactaagcaa 840
ttgcgtttgg acaccgacca agtttacggt aacattgcta cttacaactt cgaaaacggt 900
agagtcagag tcgacaccgg ttccaactgg tctgaagtct tgccacaaat ccaagaaacc 960
accgctagaa tcatcttcaa cggtaaggac ttgaacttgg ttgaaagaag aattgctgct 1020
gtcaacccat ccgacccatt ggaaaccacc aagccagaca tgaccttgaa ggaagctttg 1080
aagatcgctt tcggtttcaa cgaaccaaac ggtaacttgc aataccaagg taaggacatc 1140
accgaattcg acttcaactt cgaccaacaa acctctcaaa acatcaagaa ccaattggct 1200
gaattgaacg ctaccaacat ttacactgtt ttggacaaga tcaagttgaa cgccaagatg 1260
aacatcttga tcagagacaa gagattccac tacgacagaa acaacattgc tgtcggtgct 1320
gacgaatccg ttgtcaagga agcccacaga gaagtcatca actcctccac cgaaggtttg 1380
ttgttgaaca tcgacaagga catcagaaag atcttgtccg gttacatcgt cgaaattgaa 1440
gacaccgaag gtttgaagga agtcatcaac gacagatacg acatgttgaa catttcctct 1500
ttgagacaag acggtaagac cttcatcgac ttcaagaagt acaacgacaa gttgccattg 1560
tacatttcca acccaaacta caaggtcaac gtttacgctg tcactaagga aaacaccatc 1620
atcaacccat ccgaaaacgg tgacacttcc accaacggta tcaagaagat tttgatcttc 1680
tctaagaagg gttacgaaat tggt                                        1704
```

<210> 50
<211> 568
<212> PRT
<213> Bacillus anthracis

<400> 50

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Ser Thr Ser Ala Gly Pro Thr Val Pro Asp Arg Asp Asn Asp Gly Ile
 1               5                  10                  15
Pro Asp Ser Leu Glu Val Glu Gly Tyr Thr Val Asp Val Lys Asn Lys
            20                  25                  30
Arg Thr Phe Leu Ser Pro Trp Ile Ser Asn Ile His Glu Lys Lys Gly
        35                  40                  45
Leu Thr Lys Tyr Lys Ser Ser Pro Glu Lys Trp Ser Thr Ala Ser Asp
    50                  55                  60
Pro Tyr Ser Asp Phe Glu Lys Val Thr Gly Arg Ile Asp Lys Asn Val
65                  70                  75                  80
Ser Pro Glu Ala Arg His Pro Leu Val Ala Ala Tyr Pro Ile Val His
                85                  90                  95
Val Asp Met Glu Asn Ile Ile Leu Ser Lys Asn Glu Asp Gln Ser Thr
            100                 105                 110
Gln Asn Thr Asp Ser Glu Thr Arg Thr Ile Ser Lys Asn Thr Ser Thr
        115                 120                 125
Ser Arg Thr His Thr Ser Glu Val His Gly Asn Ala Glu Val His Ala
    130                 135                 140
Ser Phe Phe Asp Ile Gly Gly Ser Val Ser Ala Gly Phe Ser Asn Ser
145                 150                 155                 160
```

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Asn Ser Ser Thr Val Ala Ile Asp His Ser Leu Ser Leu Ala Gly Glu
                165                 170                 175
Arg Thr Trp Ala Glu Thr Met Gly Leu Asn Thr Ala Asp Thr Ala Arg
            180                 185                 190
Leu Asn Ala Asn Ile Arg Tyr Val Asn Thr Gly Thr Ala Pro Ile Tyr
        195                 200                 205
Asn Val Leu Pro Thr Thr Ser Leu Val Leu Gly Lys Asn Gln Thr Leu
    210                 215                 220
Ala Thr Ile Lys Ala Lys Glu Asn Gln Leu Ser Gln Ile Leu Ala Pro
225                 230                 235                 240
Asn Asn Tyr Tyr Pro Ser Lys Asn Leu Ala Pro Ile Ala Leu Asn Ala
                245                 250                 255
Gln Asp Asp Phe Ser Ser Thr Pro Ile Thr Met Asn Tyr Asn Gln Phe
            260                 265                 270
Leu Glu Leu Glu Lys Thr Lys Gln Leu Arg Leu Asp Thr Asp Gln Val
        275                 280                 285
Tyr Gly Asn Ile Ala Thr Tyr Asn Phe Glu Asn Gly Arg Val Arg Val
    290                 295                 300
Asp Thr Gly Ser Asn Trp Ser Glu Val Leu Pro Gln Ile Gln Glu Thr
305                 310                 315                 320
Thr Ala Arg Ile Ile Phe Asn Gly Lys Asp Leu Asn Leu Val Glu Arg
                325                 330                 335
Arg Ile Ala Ala Val Asn Pro Ser Asp Pro Leu Glu Thr Thr Lys Pro
            340                 345                 350
Asp Met Thr Leu Lys Glu Ala Leu Lys Ile Ala Phe Gly Phe Asn Glu
        355                 360                 365
Pro Asn Gly Asn Leu Gln Tyr Gln Gly Lys Asp Ile Thr Glu Phe Asp
    370                 375                 380
Phe Asn Phe Asp Gln Gln Thr Ser Gln Asn Ile Lys Asn Gln Leu Ala
385                 390                 395                 400
Glu Leu Asn Ala Thr Asn Ile Tyr Thr Val Leu Asp Lys Ile Lys Leu
                405                 410                 415
Asn Ala Lys Met Asn Ile Leu Ile Arg Asp Lys Arg Phe His Tyr Asp
            420                 425                 430
Arg Asn Asn Ile Ala Val Gly Ala Asp Glu Ser Val Val Lys Glu Ala
        435                 440                 445
His Arg Glu Val Ile Asn Ser Ser Thr Glu Gly Leu Leu Leu Asn Ile
    450                 455                 460
Asp Lys Asp Ile Arg Lys Ile Leu Ser Gly Tyr Ile Val Glu Ile Glu
465                 470                 475                 480
Asp Thr Glu Gly Leu Lys Glu Val Ile Asn Asp Arg Tyr Asp Met Leu
                485                 490                 495
Asn Ile Ser Ser Leu Arg Gln Asp Gly Lys Thr Phe Ile Asp Phe Lys
            500                 505                 510
Lys Tyr Asn Asp Lys Leu Pro Leu Tyr Ile Ser Asn Pro Asn Tyr Lys
        515                 520                 525
Val Asn Val Tyr Ala Val Thr Lys Glu Asn Thr Ile Ile Asn Pro Ser
    530                 535                 540
Glu Asn Gly Asp Thr Ser Thr Asn Gly Ile Lys Lys Ile Leu Ile Phe
545                 550                 555                 560
Ser Lys Lys Gly Tyr Glu Ile Gly
                565
```

<210> 51
<211> 420

<212> DNA
<213> Bacillus anthracis

<400> 51
ttccactacg acagaaacaa cattgctgtc ggtgctgacg aatccgttgt caaggaagcc 60
cacagagaag tcatcaactc ctccaccgaa ggtttgttgt tgaacatcga caaggacatc 120
agaaagatct tgtccggtta catcgtcgaa attgaagaca ccgaaggttt gaaggaagtc 180
atcaacgaca gatacgacat gttgaacatt tcctctttga gacaagacgg taagaccttc 240
atcgacttca agaagtacaa cgacaagttg ccattgtaca tttccaaccc aaactacaag 300
gtcaacgttt acgctgtcac taaggaaaac accatcatca acccatccga aaacggtgac 360
acttccacca acggtatcaa gaagattttg atcttctcta agaagggtta cgaaattggt 420

<210> 52
<211> 140

<212> PRT
<213> Bacillus anthracis

<400> 52
Phe His Tyr Asp Arg Asn Asn Ile Ala Val Gly Ala Asp Glu Ser Val
 1   5   10   15
Val Lys Glu Ala His Arg Glu Val Ile Asn Ser Ser Thr Glu Gly Leu
            20   25   30
Leu Leu Asn Ile Asp Lys Asp Ile Arg Lys Ile Leu Ser Gly Tyr Ile
        35   40   45
Val Glu Ile Glu Asp Thr Glu Gly Leu Lys Glu Val Ile Asn Asp Arg
    50   55   60
Tyr Asp Met Leu Asn Ile Ser Ser Leu Arg Gln Asp Gly Lys Thr Phe
65   70   75   80
Ile Asp Phe Lys Lys Tyr Asn Asp Lys Leu Pro Leu Tyr Ile Ser Asn
                85   90   95
Pro Asn Tyr Lys Val Asn Val Tyr Ala Val Thr Lys Glu Asn Thr Ile
            100   105   110
Ile Asn Pro Ser Glu Asn Gly Asp Thr Ser Thr Asn Gly Ile Lys Lys
        115   120   125
Ile Leu Ile Phe Ser Lys Lys Gly Tyr Glu Ile Gly
    130   135   140

<210> 53
<211> 2205
<212> DNA
<213> Bacillus anthracis

<400> 53
gaagttaaac aggagaaccg gttattaaat gaatcagaat caagttccca ggggttacta 60
ggatactatt ttagtgattt gaattttcaa gcacccatgg tggttacctc ttctactaca 120
ggggatttat ctattcctag ttctgagtta gaaaatattc catcggaaaa ccaatatttt 180
caatctgcta tttggtcagg atttatcaaa gttaagaaga gtgatgaata tacatttgct 240
acttccgctg ataatcatgt aacaatgtgg gtagatgacc aagaagtgat taataaagct 300
tctaattcta acaaaatcag attagaaaaa ggaagattat atcaaataaa aattcaatat 360
caacgagaaa atcctactga aaaaggattg gatttcaagt tgtactggac cgattctcaa 420
aataaaaaag aagtgatttc tagtgataac ttacaattgc cagaattaaa acaaaaatct 480
tcgaactcaa gaaaaaagcg aagtacaagt gctggaccta cggttccaga ccgtgacaat 540
gatggaatcc ctgattcatt agaggtagaa ggatatacgg ttgatgtcaa aaataaaaga 600
acttttcttt caccatggat ttctaatatt catgaaaaga aaggattaac caaatataaa 660

```
tcatctcctg aaaaatggag cacggcttct gatccgtaca gtgatttcga aaaggttaca 720
ggacggattg ataagaatgt atcaccagag gcaagacacc cccttgtggc agcttatccg 780
attgtacatg tagatatgga gaatattatt ctctcaaaaa atgaggatca atccacacag 840
aatactgata gtgaaacgag aacaataagt aaaaatactt ctacaagtag gacacatact 900
agtgaagtac atggaaatgc agaagtgcat gcgtcgttct ttgatattgg tgggagtgta 960
tctgcaggat ttagtaattc gaattcaagt acggtcgcaa ttgatcattc actatctcta 1020
gcaggggaaa gaacttgggc tgaaacaatg ggtttaaata ccgctgatac agcaagatta 1080
aatgccaata ttagatatgt aaatactggg acggctccaa tctacaacgt gttaccaacg 1140
acttcgttag tgttaggaaa aaatcaaaca ctcgcgacaa ttaaagctaa ggaaaaccaa 1200
ttaagtcaaa tacttgcacc taataattat tatccttcta aaaacttggc gccaatcgca 1260
ttaaatgcac aagacgattt cagttctact ccaattacaa tgaattacaa tcaatttctt 1320
gagttagaaa aaacgaaaca attaagatta gatacggatc aagtatatgg gaatatagca 1380
acatacaatt ttgaaaatgg aagagtgagg gtggatacag gctcgaactg gagtgaagtg 1440
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gtagaaaggc ggatagcggc ggttaatcct agtgatccat tagaaacgac taaaccggat 1560
atgacattaa aagaagccct taaaatagca tttggattta acgaaccgaa tggaaactta 1620
caatatcaag ggaaagacat aaccgaattt gattttaatt tcgatcaaca aacatctcaa 1680
aatatcaaga atcagttagc ggaattaaac gcaactaaca tatatactgt attagataaa 1740
atcaaattaa atgcaaaaat gaatatttta ataagagata aacgttttca ttatgataga 1800
aataacatag cagttggggc ggatgagtca gtagttaagg aggctcatag agaagtaatt 1860
aattcgtcaa cagagggatt attgttaaat attgataagg atataagaaa aatattatca 1920
ggttatattg tagaaattga agatactgaa gggcttaaag aagttataaa tgacagatat 1980
gatatgttga atatttctag tttacggcaa gatggaaaaa catttataga ttttaaaaaa 2040
tataatgata aattaccgtt atatataagt aatcccaatt ataaggtaaa tgtatatgct 2100
gttactaaag aaaacactat tattaatcct agtgagaatg gggatactag taccaacggg 2160
atcaagaaaa ttttaatctt ttctaaaaaa ggctatgaga tagga                  2205
```

<210> 54
<211> 735
<212> PRT
<213> Bacillus anthracis

<400> 54

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Glu Val Lys Gln Glu Asn Arg Leu Leu Asn Glu Ser Glu Ser Ser Ser
 1               5                  10                  15
Gln Gly Leu Leu Gly Tyr Tyr Phe Ser Asp Leu Asn Phe Gln Ala Pro
            20                  25                  30
Met Val Val Thr Ser Ser Thr Thr Gly Asp Leu Ser Ile Pro Ser Ser
        35                  40                  45
Glu Leu Glu Asn Ile Pro Ser Glu Asn Gln Tyr Phe Gln Ser Ala Ile
    50                  55                  60
Trp Ser Gly Phe Ile Lys Val Lys Lys Ser Asp Glu Tyr Thr Phe Ala
65                  70                  75                  80
Thr Ser Ala Asp Asn His Val Thr Met Trp Val Asp Asp Gln Glu Val
                85                  90                  95
Ile Asn Lys Ala Ser Asn Ser Asn Lys Ile Arg Leu Glu Lys Gly Arg
            100                 105                 110
Leu Tyr Gln Ile Lys Ile Gln Tyr Gln Arg Glu Asn Pro Thr Glu Lys
        115                 120                 125
Gly Leu Asp Phe Lys Leu Tyr Trp Thr Asp Ser Gln Asn Lys Lys Glu
    130                 135                 140
Val Ile Ser Ser Asp Asn Leu Gln Leu Pro Glu Leu Lys Gln Lys Ser
145                 150                 155                 160
Ser Asn Ser Arg Lys Lys Arg Ser Thr Ser Ala Gly Pro Thr Val Pro
                165                 170                 175
```

```
Asp Arg Asp Asn Asp Gly Ile Pro Asp Ser Leu Glu Val Glu Gly Tyr
            180                 185                 190
Thr Val Asp Val Lys Asn Lys Arg Thr Phe Leu Ser Pro Trp Ile Ser
        195                 200                 205
Asn Ile His Glu Lys Lys Gly Leu Thr Lys Tyr Lys Ser Ser Pro Glu
    210                 215                 220
Lys Trp Ser Thr Ala Ser Asp Pro Tyr Ser Asp Phe Glu Lys Val Thr
225                 230                 235                 240
Gly Arg Ile Asp Lys Asn Val Ser Pro Glu Ala Arg His Pro Leu Val
                245                 250                 255
Ala Ala Tyr Pro Ile Val His Val Asp Met Glu Asn Ile Ile Leu Ser
            260                 265                 270
Lys Asn Glu Asp Gln Ser Thr Gln Asn Thr Asp Ser Glu Thr Arg Thr
        275                 280                 285
Ile Ser Lys Asn Thr Ser Thr Ser Arg Thr His Thr Ser Glu Val His
    290                 295                 300
Gly Asn Ala Glu Val His Ala Ser Phe Phe Asp Ile Gly Gly Ser Val
305                 310                 315                 320
Ser Ala Gly Phe Ser Asn Ser Asn Ser Ser Thr Val Ala Ile Asp His
                325                 330                 335
Ser Leu Ser Leu Ala Gly Glu Arg Thr Trp Ala Glu Thr Met Gly Leu
            340                 345                 350
Asn Thr Ala Asp Thr Ala Arg Leu Asn Ala Asn Ile Arg Tyr Val Asn
        355                 360                 365
Thr Gly Thr Ala Pro Ile Tyr Asn Val Leu Pro Thr Thr Ser Leu Val
    370                 375                 380
Leu Gly Lys Asn Gln Thr Leu Ala Thr Ile Lys Ala Lys Glu Asn Gln
385                 390                 395                 400
Leu Ser Gln Ile Leu Ala Pro Asn Asn Tyr Tyr Pro Ser Lys Asn Leu
                405                 410                 415
Ala Pro Ile Ala Leu Asn Ala Gln Asp Asp Phe Ser Ser Thr Pro Ile
            420                 425                 430
Thr Met Asn Tyr Asn Gln Phe Leu Glu Leu Glu Lys Thr Lys Gln Leu
        435                 440                 445
Arg Leu Asp Thr Asp Gln Val Tyr Gly Asn Ile Ala Thr Tyr Asn Phe
    450                 455                 460
Glu Asn Gly Arg Val Arg Val Asp Thr Gly Ser Asn Trp Ser Glu Val
465                 470                 475                 480
Leu Pro Gln Ile Gln Glu Thr Thr Ala Arg Ile Ile Phe Asn Gly Lys
                485                 490                 495
Asp Leu Asn Leu Val Glu Arg Arg Ile Ala Ala Val Asn Pro Ser Asp
            500                 505                 510
Pro Leu Glu Thr Thr Lys Pro Asp Met Thr Leu Lys Glu Ala Leu Lys
        515                 520                 525
Ile Ala Phe Gly Phe Asn Glu Pro Asn Gly Asn Leu Gln Tyr Gln Gly
    530                 535                 540
Lys Asp Ile Thr Glu Phe Asp Phe Asn Phe Asp Gln Gln Thr Ser Gln
545                 550                 555                 560
Asn Ile Lys Asn Gln Leu Ala Glu Leu Asn Ala Thr Asn Ile Tyr Thr
                565                 570                 575
Val Leu Asp Lys Ile Lys Leu Asn Ala Lys Met Asn Ile Leu Ile Arg
            580                 585                 590
Asp Lys Arg Phe His Tyr Asp Arg Asn Asn Ile Ala Val Gly Ala Asp
        595                 600                 605
```

```
Glu Ser Val Val Lys Glu Ala His Arg Glu Val Ile Asn Ser Ser Thr
    610                 615                 620
Glu Gly Leu Leu Leu Asn Ile Asp Lys Asp Ile Arg Lys Ile Leu Ser
625                 630                 635                 640
Gly Tyr Ile Val Glu Ile Glu Asp Thr Glu Gly Leu Lys Glu Val Ile
                645                 650                 655
Asn Asp Arg Tyr Asp Met Leu Asn Ile Ser Ser Leu Arg Gln Asp Gly
            660                 665                 670
Lys Thr Phe Ile Asp Phe Lys Lys Tyr Asn Asp Lys Leu Pro Leu Tyr
        675                 680                 685
Ile Ser Asn Pro Asn Tyr Lys Val Asn Val Tyr Ala Val Thr Lys Glu
    690                 695                 700
Asn Thr Ile Ile Asn Pro Ser Glu Asn Gly Asp Thr Ser Thr Asn Gly
705                 710                 715                 720
Ile Lys Lys Ile Leu Ile Phe Ser Lys Lys Gly Tyr Glu Ile Gly
                725                 730                 735
```

<210> 55
<211> 4365
<212> DNA
<213> Bacillus anthracis

<400> 55

```
gaagttaagc aagaaaacag attgttgaac gaatctgaat cctcttccca aggtttgttg 60
cttcaattcg ttcttttgtc taacaacttg cttagactta ggagaagggt tccaaacaac 120
ggttactact tctccgactt gaacttccaa gctccaatgg tcgtcacctc ctctactacc 180
ccaatgatga agaggctgaa cttgaaggtt cgaggttacc agcagtggag gagatgatgg 240
ggtgacttgt ccatcccatc ctccgaatta gagaacatcc catccgaaaa ccaatacttc 300
ccactgaaca ggtagggtag gaggcttaat ctcttgtagg gtaggctttt ggttatgaag 360
caatccgcta tctggtccgg cttcatcaag gtcaagaagt ccgacgaata cactttcgct 420
gttaggcgat agaccaggcc gaagtagttc cagttcttca ggctgcttat gtgaaagcga 480
acctccgctg acaaccacgt tactatgtgg gtcgacgacc aagaagtcat caacaaggct 540
tggaggcgac tgttggtgca atgatacacc cagctgctgg ttcttcagta gttgttccga 600
tccaactcca acaagatcag attggaaaag ggtagattgt accaaattaa gatccaatac 660
aggttgaggt tgttctagtc taacctttc ccatctaaca tggtttaatt ctaggttatg 720
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aacaagaagg aagtcatctc ctctgacaac ttgcaattgc cagaattgaa gcaaaagtcc 900
ttgttcttcc ttcagtagag gagactgttg aacgttaacg gtcttaactt cgttttcagg 960
tccaactcca gaaagaagag atccacctcc gctggtccaa ctgttccaga cagagacaac 1020
aggttgaggt ctttcttctt caggtggagg cgaccaggtt gacaaggtct gtctctgttg 1080
gacggtattc cagactcctt ggaagtcgaa ggttacaccg tcgacgtcaa gaacaagaga 1140
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<210> 56
<211> 735
<212> PRT
<213> Bacillus anthracis

<400> 56

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Met Val Val Thr Ser Ser Thr Thr Gly Asp Leu Ser Ile Pro Ser Ser
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Glu Leu Glu Asn Ile Pro Ser Glu Asn Gln Tyr Phe Gln Ser Ala Ile
    50                  55                  60
Trp Ser Gly Phe Ile Lys Val Lys Lys Ser Asp Glu Tyr Thr Phe Ala
65                  70                  75                  80
Thr Ser Ala Asp Asn His Val Thr Met Trp Val Asp Asp Gln Glu Val
                85                  90                  95
Ile Asn Lys Ala Ser Asn Ser Asn Lys Ile Arg Leu Glu Lys Gly Arg
            100                 105                 110
Leu Tyr Gln Ile Lys Ile Gln Tyr Gln Arg Glu Asn Pro Thr Glu Lys
        115                 120                 125
Gly Leu Asp Phe Lys Leu Tyr Trp Thr Asp Ser Gln Asn Lys Lys Glu
    130                 135                 140
Val Ile Ser Ser Asp Asn Leu Gln Leu Pro Glu Leu Lys Gln Lys Ser
145                 150                 155                 160
Ser Asn Ser Arg Lys Lys Arg Ser Thr Ser Ala Gly Pro Thr Val Pro
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Asp Arg Asp Asn Asp Gly Ile Pro Asp Ser Leu Glu Val Glu Gly Tyr
            180                 185                 190
Thr Val Asp Val Lys Asn Lys Arg Thr Phe Leu Ser Pro Trp Ile Ser
        195                 200                 205
Asn Ile His Glu Lys Lys Gly Leu Thr Lys Tyr Lys Ser Ser Pro Glu
    210                 215                 220
Lys Trp Ser Thr Ala Ser Asp Pro Tyr Ser Asp Phe Glu Lys Val Thr
225                 230                 235                 240
Gly Arg Ile Asp Lys Asn Val Ser Pro Glu Ala Arg His Pro Leu Val
                245                 250                 255
Ala Ala Tyr Pro Ile Val His Val Asp Met Glu Asn Ile Ile Leu Ser
            260                 265                 270
Lys Asn Glu Asp Gln Ser Thr Gln Asn Thr Asp Ser Glu Thr Arg Thr
        275                 280                 285
Ile Ser Lys Asn Thr Ser Thr Ser Arg Thr His Thr Ser Glu Val His
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Gly Asn Ala Glu Val His Ala Ser Phe Phe Asp Ile Gly Gly Ser Val
305                 310                 315                 320
Ser Ala Gly Phe Ser Asn Ser Asn Ser Ser Thr Val Ala Ile Asp His
                325                 330                 335
Ser Leu Ser Leu Ala Gly Glu Arg Thr Trp Ala Glu Thr Met Gly Leu
            340                 345                 350
Asn Thr Ala Asp Thr Ala Arg Leu Asn Ala Asn Ile Arg Tyr Val Asn
        355                 360                 365
Thr Gly Thr Ala Pro Ile Tyr Asn Val Leu Pro Thr Thr Ser Leu Val
    370                 375                 380
Leu Gly Lys Asn Gln Thr Leu Ala Thr Ile Lys Ala Lys Glu Asn Gln
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Leu Ser Gln Ile Leu Ala Pro Asn Asn Tyr Tyr Pro Ser Lys Asn Leu
                405                 410                 415
Ala Pro Ile Ala Leu Asn Ala Gln Asp Asp Phe Ser Ser Thr Pro Ile
            420                 425                 430
Thr Met Asn Tyr Asn Gln Phe Leu Glu Leu Glu Lys Thr Lys Gln Leu
        435                 440                 445
Arg Leu Asp Thr Asp Gln Val Tyr Gly Asn Ile Ala Thr Tyr Asn Phe
    450                 455                 460
Glu Asn Gly Arg Val Arg Val Asp Thr Gly Ser Asn Trp Ser Glu Val
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Asp Leu Asn Leu Val Glu Arg Arg Ile Ala Ala Val Asn Pro Ser Asp
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Pro Leu Glu Thr Thr Lys Pro Asp Met Thr Leu Lys Glu Ala Leu Lys
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Ile Ala Phe Gly Phe Asn Glu Pro Asn Gly Asn Leu Gln Tyr Gln Gly
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Lys Asp Ile Thr Glu Phe Asp Phe Asn Phe Asp Gln Gln Thr Ser Gln
545                 550                 555                 560
Asn Ile Lys Asn Gln Leu Ala Glu Leu Asn Ala Thr Asn Ile Tyr Thr
                565                 570                 575
Val Leu Asp Lys Ile Lys Leu Asn Ala Lys Met Asn Ile Leu Ile Arg
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Asp Lys Arg Phe His Tyr Asp Arg Asn Asn Ile Ala Val Gly Ala Asp
        595                 600                 605
Glu Ser Val Val Lys Glu Ala His Arg Glu Val Ile Asn Ser Ser Thr
    610                 615                 620
Glu Gly Leu Leu Leu Asn Ile Asp Lys Asp Ile Arg Lys Ile Leu Ser
625                 630                 635                 640
Gly Tyr Ile Val Glu Ile Glu Asp Thr Glu Gly Leu Lys Glu Val Ile
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Asn Asp Arg Tyr Asp Met Leu Asn Ile Ser Ser Leu Arg Gln Asp Gly
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Lys Thr Phe Ile Asp Phe Lys Lys Tyr Asn Asp Lys Leu Pro Leu Tyr
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Ile Ser Asn Pro Asn Tyr Lys Val Asn Val Tyr Ala Val Thr Lys Glu
    690                 695                 700
Asn Thr Ile Ile Asn Pro Ser Glu Asn Gly Asp Thr Ser Thr Asn Gly
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Ile Lys Lys Ile Leu Ile Phe Ser Lys Lys Gly Tyr Glu Ile Gly
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<210> 57
<211> 4365
<212> DNA
<213> Bacillus anthracis

<400> 57

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<210> 58
<211> 735
<212> PRT
<213> Bacillus anthracis

<400> 58

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			20					25					30		
Met	Val	Val	Thr	Ser	Ser	Thr	Thr	Gly	Asp	Leu	Ser	Ile	Pro	Ser	Ser
		35					40					45			
Glu	Leu	Glu	Asn	Ile	Pro	Ser	Glu	Asn	Gln	Tyr	Phe	Gln	Ser	Ala	Ile
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65					70					75					80
Thr	Ser	Ala	Asp	Asn	His	Val	Thr	Met	Trp	Val	Asp	Asp	Gln	Glu	Val
				85					90					95	
Ile	Asn	Lys	Ala	Ser	Asn	Ser	Asn	Lys	Ile	Arg	Leu	Glu	Lys	Gly	Arg
			100					105					110		
Leu	Tyr	Gln	Ile	Lys	Ile	Gln	Tyr	Gln	Arg	Glu	Asn	Pro	Thr	Glu	Lys
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Val	Ile	Ser	Ser	Asp	Asn	Leu	Gln	Leu	Pro	Glu	Leu	Lys	Gln	Lys	Ser
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Ser	Asn	Ser	Arg	Lys	Lys	Lys	Ser	Thr	Ser	Ala	Gly	Pro	Thr	Val	Pro
				165					170					175	
Asp	Arg	Asp	Asn	Asp	Gly	Ile	Pro	Asp	Ser	Leu	Glu	Val	Glu	Gly	Tyr
			180					185					190		
Thr	Val	Asp	Val	Lys	Asn	Lys	Arg	Thr	Phe	Leu	Ser	Pro	Trp	Ile	Ser
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Asn	Ile	His	Glu	Lys	Lys	Gly	Leu	Thr	Lys	Tyr	Lys	Ser	Ser	Pro	Glu
	210					215					220				
Lys	Trp	Ser	Thr	Ala	Ser	Asp	Pro	Tyr	Ser	Asp	Phe	Glu	Lys	Val	Thr
225					230					235					240
Gly	Arg	Ile	Asp	Lys	Asn	Val	Ser	Pro	Glu	Ala	Arg	His	Pro	Leu	Val
				245					250					255	
Ala	Ala	Tyr	Pro	Ile	Val	His	Val	Asp	Met	Glu	Asn	Ile	Ile	Leu	Ser
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Lys	Asn	Glu	Asp	Gln	Ser	Thr	Gln	Asn	Thr	Asp	Ser	Glu	Thr	Arg	Thr
		275					280					285			
Ile	Ser	Lys	Asn	Thr	Ser	Thr	Ser	Arg	Thr	His	Thr	Ser	Glu	Val	His
	290					295					300				
Gly	Asn	Ala	Glu	Val	His	Ala	Ser	Phe	Phe	Asp	Ile	Gly	Gly	Ser	Val
305					310					315					320
Ser	Ala	Gly	Phe	Ser	Asn	Ser	Asn	Ser	Ser	Thr	Val	Ala	Ile	Asp	His
				325					330					335	
Ser	Leu	Ser	Leu	Ala	Gly	Glu	Arg	Thr	Trp	Ala	Glu	Thr	Met	Gly	Leu
			340					345					350		
Asn	Thr	Ala	Asp	Thr	Ala	Arg	Leu	Asn	Ala	Asn	Ile	Arg	Tyr	Val	Asn
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Leu Ser Gln Ile Leu Ala Pro Asn Asn Tyr Tyr Pro Ser Lys Asn Leu
                405                 410                 415
Ala Pro Ile Ala Leu Asn Ala Gln Asp Asp Phe Ser Ser Thr Pro Ile
            420                 425                 430
Thr Met Asn Tyr Asn Gln Phe Leu Glu Leu Glu Lys Thr Lys Gln Leu
        435                 440                 445
Arg Leu Asp Thr Asp Gln Val Tyr Gly Asn Ile Ala Thr Tyr Asn Phe
    450                 455                 460
Glu Asn Gly Arg Val Arg Val Asp Thr Gly Ser Asn Trp Ser Glu Val
465                 470                 475                 480
Leu Pro Gln Ile Gln Glu Thr Thr Ala Arg Ile Ile Phe Asn Gly Lys
                485                 490                 495
Asp Leu Asn Leu Val Glu Arg Arg Ile Ala Ala Val Asn Pro Ser Asp
            500                 505                 510
Pro Leu Glu Thr Thr Lys Pro Asp Met Thr Leu Lys Glu Ala Leu Lys
        515                 520                 525
Ile Ala Phe Gly Phe Asn Glu Pro Asn Gly Asn Leu Gln Tyr Gln Gly
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Lys Asp Ile Thr Glu Phe Asp Phe Asn Phe Asp Gln Gln Thr Ser Gln
545                 550                 555                 560
Asn Ile Lys Asn Gln Leu Ala Glu Leu Asn Ala Thr Asn Ile Tyr Thr
                565                 570                 575
Val Leu Asp Lys Ile Lys Leu Asn Ala Lys Met Asn Ile Leu Ile Arg
            580                 585                 590
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        595                 600                 605
Glu Ser Val Val Lys Glu Ala His Arg Glu Val Ile Asn Ser Ser Thr
    610                 615                 620
Glu Gly Leu Leu Leu Asn Ile Asp Lys Asp Ile Arg Lys Ile Leu Ser
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Gly Tyr Ile Val Glu Ile Glu Asp Thr Glu Gly Leu Lys Glu Val Ile
                645                 650                 655
Asn Asp Arg Tyr Asp Met Leu Asn Ile Ser Ser Leu Arg Gln Asp Gly
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Lys Thr Phe Ile Asp Phe Lys Lys Tyr Asn Asp Lys Leu Pro Leu Tyr
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Ile Ser Asn Pro Asn Tyr Lys Val Asn Val Tyr Ala Val Thr Lys Glu
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Asn Thr Ile Ile Asn Pro Ser Glu Asn Gly Asp Thr Ser Thr Asn Gly
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<210> 59
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<212> DNA
<213> Bacillus anthracis

<400> 59
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<210> 60
<211> 29
<212> DNA
<213> Bacillus anthracis

<400> 60
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<210> 61
<211> 46
<212> DNA
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